The Effects of Enhanced UV-B on Plant Competition:

An Application of Metabolic Fingerprinting

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By

George Rinu

University of Wales, Aberystwyth

Institute of Biology

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Abstract

Concerns about increased stratospheric ozone depletion increasing ambient levels of ultraviolet-B radiation (UV-B), and the fact that some ecosystems are naturally exposed to high levels, has resulted in an increased interest in the effects of UV-B on plant communities. Despite this, there has been a paucity of studies into its effects on plant competition. Artificial plant communities consisting of *Lolium perenne* and *Lotus corniculatus* and a sub-montane community consisting of *Agrostis tenuis*, *Festuca ovina* and *Galium saxatile* (also including different nitrogen levels) were created using the response surface approach. The long-term effects of UV-B were also studied on a natural sub-Arctic community in Abisko, Sweden. In addition, all plant samples were analysed by Fourier-Transform Infrared Spectroscopy (FT-IR) to obtain a 'metabolic fingerprint' which was used to detect chemical differences to the whole biochemical complement of the sample. The results showed that enhanced UV-B altered the competitive interaction of *Lolium perenne* and *Lotus corniculatus* in favour of *Lolium perenne* although ambient levels of UV-B did not elicit an effect in the sub-montane community. Only one dwarf shrub species in the sub-Arctic experiment, *Vaccinium myrtillus*, was negatively affected by UV-B. In most cases, elevated UV-B elicited a change in the metabolic fingerprint in the samples and in some cases an alteration in competitive stress altered the metabolome. This suggests that FT-IR can be used as a screening tool to detect for both abiotic stress and competitive biochemical alterations. In addition, this thesis proposes that the facilitative effect between the grass-legume mixture of *Lolium perenne* and *Lotus corniculatus* is not related to nitrogen fixation in the early stages of competition which has traditionally been believed.

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Chapter One: Introduction

1.1: *Overview*

The definition of stress in plant ecology has been subject to much debate although the consensus is that a stress is any external condition which limits optimum biomass being obtained (Grime, 1979). Opponents to this view suggest that this overemphasises the role of biomass as an indicator of health with fecundity being perhaps more appropriate (Silvertown & Lovett-Doust, 1993). For example, most plant populations are hindered by a lack of resources yet they are still reproductively viable (Groth *et al.*, 1996). Nonetheless, it is clear that factors commonly regarded as stresses such as enhanced ultraviolet radiation (Björn *et al.*, 1997), high salinity (Hasegawa & Bressan, 2000) and extreme temperature variation (Shinozaki & Yamaguchi-Shinozaki, 2000) all have a negative effect on biomass. Furthermore, biomass is often correlated to reproductive fitness which is another advantage of using this approach (Weiher *et al.*, 1999). For these reasons, this project will take the view that biomass is an appropriate indicator of stress.

There has also been much discussion into the most appropriate way to classify different types of stress (Grace, 1991). One common delineation is to classify according to biotic stress (for example, competition and herbivory) and abiotic stress (for example, toxicity and nutrient deficiency) (Creelman & Mullet, 1993). Biotic stress from plant competition is one of the most ubiquitous stresses as communities are by definition assemblages of species sharing common resources (Smith & Huston, 2004). This project aims to study the intersection of these two types of stress by

studying the effect of ultraviolet-B (UV-B) radiation (an abiotic stress) on plant competition (a biotic stress).

UV-B radiation is one of the most widely studied types of plant stress (Rozema *et al.*, 1997a) and has been used extensively in both glasshouse and outdoor experiments (Newsham *et al.*, 1996). This has been driven by concerns for ozone depletion elevating ambient UV-B levels (Frederick & Snell, 1988; Gleason *et al*., 1993; Müller *et al*., 1997) although revisionist theorists emphasise the importance of UV-B as an abiotic stress that would be significant even in the absence of ozone depletion (Paul, 2001; Paul & Gwynn Jones, 2003; Aphalo 2003). Most studies show that UV-B decreases biomass although the extent is dependent on the species (Björn *et al.*, 1997). For this reason, it can be hypothesised that UV-B will alter the competitive balance of a plant community in the favour of those species more able to tolerate the stress. Despite, the widespread study of UV-B in plants there have been relatively few studies directly studying the effects of UV-B on plant competition (Barnes *et al.*, 1995, 1996). Furthermore, these studies used replacement series designs which are currently being replaced by more advanced designs such as surface response analysis (Gibson *et al.*, 1999). This thesis aims to expand the knowledge in this area by using these more advanced designs.

Additionally, the effects of UV-B on plant competition have never been directly analysed in the conflicting theories of Tilman (1985) and Grime (1977). This is surprising as their debate has been the focus for most of the theoretical studies into the effects of stress on plant competition (Grace, 1991). Grime suggested that plant competition only occurs when there is absence of stress (Grime, 1989) whilst Tilman

argued that competition is always present regardless of stress (Tilman, 1987; 1990). As with the effects of UV-B on plant competition, there has been a paucity of empirical studies testing these hypotheses and this thesis aims to redress this balance by using a series of competition experiments using three types of plant community.

The first type of community was an artificial grass-legume system consisting of *Lolium perenne* and *Lotus corniculatus*. In some experiments the subspecies *L. corniculatus var. japonicus* was used and is commonly referred to as *L. japonicus* for ease of use (Ito *et al.*, 2004; Márquez *et al.*, 2005). Mixtures of grasses and legumes are often studied as they often overyield (where the mixtures are more productive than the monocultures) which is of clear agronomic (Niang *et al.*, 1998) and ecological importance (Springer *et al.*, 2001). The second system was an artificial sub-montane grassland community based on community U4 of the National Vegetation Classification scheme (Hulme *et al*., 1999; Milne *et al*., 2002). Such communities are also subject to nitrogen deposition pollution (Lee, 1998) and because of this the experiment had the additional factor of variable nitrogen levels. The final experiment investigated the effect of UV-B on a sub-arctic heath where there are concerns about enhanced UV-B affecting plant communities (Gwynn Jones *et al.*, 1999a). The final analysis aims to draw conclusions from all the communities to see whether there are any unifying effects of stress on plant communities.

In addition, this thesis introduces the novel application of metabolic fingerprinting to stress-competition research. Metabolomics (of which metabolic fingerprinting is a branch) is concerned with holistically analysing the entire biochemical complement of a sample (the metabolome) simultaneously (Fiehn, 2002). This differs from

traditional biochemistry where only one chemical group would be identified in isolation from the others (Weckworth, 2003). The branch of metabolic fingerprinting utilises technologies such as Mass Spectrometry (MS) and Fourier Transform Infrared Spectroscopy (FT-IR) to classifying and identifying samples according to their metabolome (Goodacre *et al.*, 1996). In stress research it could be hypothesised that the chemical alterations induced by the stress can be detected by metabolic fingerprinting technologies such as FT-IR. This would then provide the opportunity to ascertain the extent of stress from specific chemical signatures. Metabolic fingerprinting by FT-IR is therefore used in all the experiments to see whether the effects of stress and competition are detectable in the metabolome.

The following sections of the introduction elaborate upon the central hypotheses of the thesis. The reasons for studying UV-B are initially justified and this is followed by an introduction of the Tilman-Grime debate with reference to how UV-B research could help reach a conclusion. A hypothesis is then presented which suggests that UV-B could indirectly alter plant interactions via shifts in soil leachates. This is followed by a discussion of metabolic fingerprinting and how this could be used to detect UV-B mediated alterations to both soil leachates and plant material. The introduction then concludes with an overview of the main hypotheses.

1.2: *Ambient UV-B as an Abiotic Stress*

The past two decades has seen an increase in the number of studies pertaining to the effects of ultraviolet radiation on plants. Invariably, the reason for these studies has been concern about stratospheric ozone depletion increasing ambient levels of potentially damaging UV-B radiation. However, it has recently been highlighted that such a justification misleadingly overemphasizes the role of stratospheric ozone in determining ambient levels and ignores the fact that other factors determine UV-B levels (Paul, 2001; Paul & Gwynn Jones, 2003; Aphalo 2003).

Figure 1 shows the main determinants of ambient UV-B. UV-B predictably alters with the time of day with midday showing the greatest levels (Figure 1a). Geographic location is also important as the solar zenith angle (SZA) is a key factor in determining UV-B levels (Figure 1b). At low solar elevations the path length of radiation is longer thus increasing the absorbance by stratospheric ozone (McKenzie *et al.*, 2003). For this reason, land closer to the equator receives greater levels of UV-B radiation. Altitude is also important (Figure 1c) with increases of 2-23% ambient UV-B per kilometre of altitude (Varotsos *et al.*, 2001). In some cases the effect of altitude outweighs that of geographic location. Tallahassee, FL (30.4°N) receives less UV-B than Albuquerque, NM (35.0°N), despite being more southerly, due to its lower elevation (2 metres compared to 1619 metres) (Scotto *et al.*, 1988).

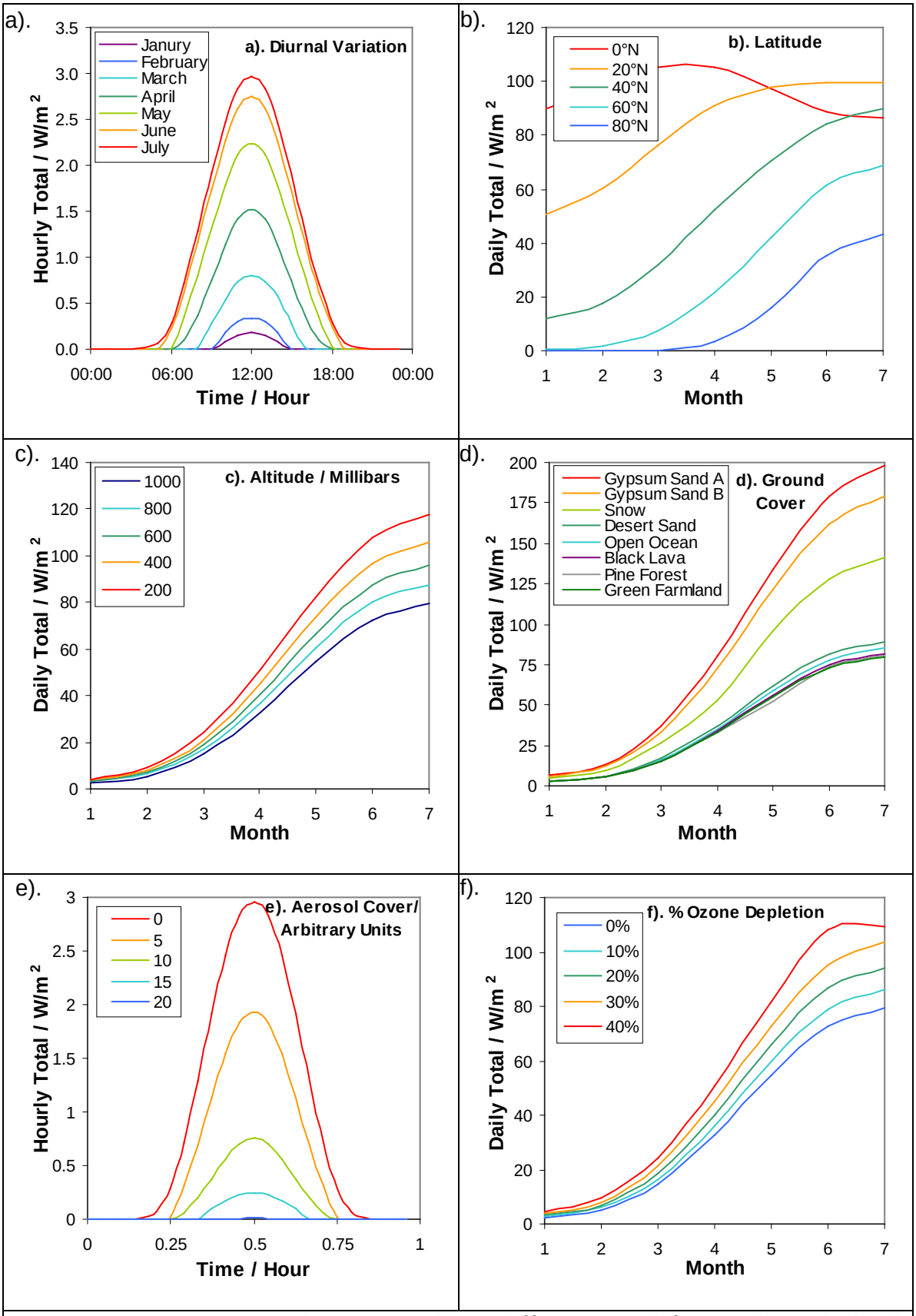


Figure 1.1. Variation in ambient UV-B due to different natural factors (data calculated from models by Björn & Murphy, 1985).

UV-B can also alter due to other variable environmental factors regardless of geographic location and altitude. For example, the type of ground cover is a key determinant of ambient UV-B (Figure 1d) with the majority of research concentrating on the surface albedo of snow which substantially increases UV-B (McKenzie et al., 1998; Degünther and Meerkötter, 2000). Cloud cover is also fundamental to ambient UV-B with complete absence under overcast conditions (Bais *et al.*, 1993). Artificial aerosols similarly attenuate UV-B (Figure 1e) and urban pollution is a key factor in determining the UV-B levels in large conurbations such as Mexico City (Acosta et al., 2000) and the east coast cities of the United States (Wenny et al., 1998; 2001).

Therefore, UV-B is an important environmental factor which has the potential to vastly alter ecosystems regardless of whether stratospheric ozone depletion increases ambient levels (Paul, 2001). It is therefore ecologically relevant and easily administered under experimental conditions due to the widespread availability of UV-B emitting lamps. All the experiments in this thesis utilise UV-B to ascertain the effects of stress on plant interactions. The main theories which relate to the effects of stress on plant competition are outlined in the next section regarding the debate between Tilman and Grime.

1.3: *The Tilman-Grime Debate*

The role of abiotic stress in altering plant competition is one of the most widely debated areas in ecology. The debate focuses on the conflicting theories of David Tilman (1987; 1990) and Phil Grime (1974; 1989). Whilst the debate encompasses many disparate areas of research, the essence of the debate can be distilled into two conflicting hypotheses: (1) That competition is important regardless of stress (Tilman, 1985) and (2) competition is only important in the absence of stress (Grime, 1977).

Grime's theory is essentially an extension of his novel plant strategy work (Grime, 1974; Grime *et al*., 1988). Three key plant strategies are identified: competitors (C), stress-tolerators (S) and ruderals (R). The three strategies are represented at the vertices of a triangle with a specific plant's strategy being a trade-off between the three functional types that best represents the ecological niche. For example, under high nutrient and low disturbance conditions, plants that can obtain nutrients more effectively and perpetuate their dominance through organs of perennation will persist (the so-called competitors). Under low nutrient and low disturbance environments (in terms of physical disruption), this tactic would not be viable and only plants whose resources are dedicated to stress-tolerance would survive (the so-called stress-tolerators). In high nutrient and high disturbance environments, neither of these strategies would be viable allowing fast-growing species with short life-spans to persist (the so-called ruderals).

It is from this background that Grime concluded that competition would only be present in high-nutrient conditions (Grime, 1977; 1989). According to this theory,

those species which are effective competitors cannot thrive under low-nutrient conditions as their resources are dedicated to competing and not tolerating stress. In such conditions they would not survive allowing stress-tolerators to persist. Whilst there is a clear outcome (the dominance of stress-tolerators) this would not be because of direct competition for resources from the competitors. Conversely, a stress-tolerator would not survive in a nutrient-rich habitat as its resources are not dedicated to capturing nutrients. Whilst the logic of the hypothesis is sound it is solely dependent on the assumption that three-primary strategies exist. This has proved contentious (Loehle, 1988; see Grime, 1988, for response) and it is suggested that more experimental work is needed (Campbell *et al.*, 1991).

On the other hand, Tilman's Resource Ratio (Tilman, 1985; 1987; 1990; Pacala & Tilman, 1994) provides the basis for the contention that competition always occurs regardless of stress. The theory essentially states that one species in a mixed community will be more efficient at depleting nutrient levels than the others. Over time, the nutrients will be depleted below the level which other species can tolerate and thus one species will dominate. However, when nutrient conditions are plentiful, Tilman states that competition will shift from belowground to aboveground for light. Under these conditions, the species that can maximise light-acquisition will dominate. This remains controversial as it suggests that competition is important under conditions of low abiotic stress which is the opposite of what Grime predicted. Therefore, in Tilman's view, abiotic stress does not negate competition but simply alters the form.

The debate has been extensively reviewed (Grace, 1993; Grubb *et al.*, 1997; Aerts, 1999) and has occasionally become heated with criticism of national bias being made (Rydin and Bengtsson, 1990). Despite some notable attempts at reconciliation by Grace (1991; 1995) the debate remains open.

The lack of closure in the debate is partially due to a lack of empirical evidence to disprove either theory (Wilson & Lee, 2000). The debate has primarily been theoretical with a paucity of direct tests. Another reason for the lack of experimental interest is possibly that the two protagonists have switched their attention to issues of biodiversity in ecosystem stability; a debate that has proven even more controversial than the original. Tilman maintains that biodiversity is essential for ecosystem stability (Tilman, 1999; Tilman *et al.*, 2006) whilst Grime states that increased biodiversity is not necessarily beneficial for an ecosystem (Grime, 1997; Wardle & Grime, 2003). However, with climate change being of current importance, Brooker (2006) has asserted that the need to re-open the original debate has never been more pressing.

Whilst UV-B is widely regarded as being an ubiquitous plant stress (Rozema *et al.*, 1997b; Mackerness, 2000; Jordan, 2002) it has seldom been used in plant competition experiments and never in the context of the Tilman-Grime debate. There have been numerous studies investigating the effects of UV-B on natural communities (Björn *et al.*, 1997; Gwynn Jones *et al.*, 1997; Ballaré *et al.*, 1997) although the central concern has been on the outcome of the stress and not on the mechanistic basis underpinning the interactions. Such an aim would prove difficult given that natural communities

were used. This thesis therefore aims to resolve the gaps in the literature by testing the effects of UV-B on an experiment designed to investigate competitive interactions.

There are numerous reasons to hypothesise why UV-B could alter competitive interactions. It is clear that the effects of UV-B differ depending on plant species. Numerous studies have investigated the effects of UV-B on a range of related species such as agricultural weeds (Furness & Upadhyaya, 2002), rice cultivars (Dai *et al.*, 1992; Dai *et al.*, 1998) and maize strains (Correia *et al.*, 1998). All the studies point towards a broad spectrum of negative to positive responses. When extrapolated to an ecological context it becomes clear that in a high UV-B environment, species that can tolerate UV-B will be more dominant (given Grime's theory). Moreover, research from Barnes *et al.* (1990 a, 1993) suggests that the greatest effects of UV-B are not reductions in biomass but morphological characteristics. This could alter canopy structure which in turn could have profound effects on competition for light.

By employing enhanced UV-B as a stress on plant communities, this thesis aims to further the empirical work conducted into the theories of Tilman and Grime by testing the hypothesis that UV-B will alter plant communities in favour of those species more able to tolerate the stress. Whilst this hypothesis is based on the direct affects of UV-B on the individual plants, it is also possible that UV-B could indirectly alter plant interactions via changes to belowground parameters. The details of this hypothesis are presented in the following section.

1.4: *The Role of Soil Leachates in Mediating Plant Interactions*

Another reason to hypothesise why UV-B may alter community structure is that UV-B has been shown to affect belowground processes such as microbial communities (Johnson *et al.*, 2002) and mycorrhizae formation (van de Staaij *et al.*, 2001; Zaller *et al.*, 2002). This is surprising given that UV-B cannot directly penetrate the soil (Green, 1983) suggesting the effects are likely to be plant-mediated. There is evidence to suggest that litter degradation is altered through a combination of direct exposure and altered leaf chemistry (Gehrke *et al.*, 1995). However, evidence also suggests that UV-B is affecting the quality of root exudates, which is having an effect on microbial communities (Shiozaki *et al.*, 1999; Pinto *et al.*, 2002). One hypothesis for this effect is that UV-B is known to increase flavonoid compounds in the roots (Shirley, 1996) and the same compounds are involved in forming belowground symbiotic relations (Redmond *et al.*, 1986). Study of soil leachates by metabolomics could help test this hypothesis. Therefore, in the majority of experiments used in this thesis, leachates were collected from the plant communities and analysed to test for any changes induced by both plant community type and UV-B. The method used to detect any differences was metabolic fingerprinting by FT-IR and the rationale for this technique is introduced in the following section.

1.5: *Metabolic Fingerprinting*

The metabolome can be defined as the total biochemical complement of a cell or particular organ (Fiehn, 2001). Technological advances in equipment such as mass and infrared spectroscopes have allowed the complete metabolome to be analysed simultaneously for the first time (Sumner *et al.*, 2003). This has the potential to complement the other 'omic' fields of biology such as genomics, proteomics and transcriptomics; ultimately leading to a more comprehensive knowledge of organism functioning (Weckworth, 2003). Furthermore, the field of metabolomics, predominantly via the use of metabolomic fingerprinting, provides a powerful tool allowing rapid and potentially informative snapshots of the interaction between the environment and a plant's biochemistry.

The importance of metabolomics lies in the fact that the metabolome is the ultimate product of the genome. Much research has been carried out to sequence the genomes of various organisms with bioinformatic studies mining the data for similarities in sequences to provide information into the function of genetic material. Further 'omic' strategies include proteomics and transcriptomics that aim to find the total complement of proteins or mRNA molecules encoded by the genome respectively. However, if the genome is to be of any practical use it is crucial that the ultimate effects are ascertained which will be manifested via the metabolome (Sumner *et al.*, 2003). For example. It is necessary to understand how a new drug will affect the whole organism and not just the organs to which it is targeted (Kenney & Shockor, 2003). The effect of an introduced transgene to a plant cannot be ascertained by knowledge of how the gene functions by itself, as it may affect the whole metabolome;

this is of particular importance to address public concerns about the safety of transgenic crops (Fiehn *et al.*, 2000).

Traditionally, biochemical research has tended to concentrate on one specific metabolite (metabolic target analysis) or a specific group of chemicals such as amino acids or phenyl-propanoids (metabolite profiling) (Weckworth, 2003). Recent advances, namely with mass spectroscopy, have allowed a more holistic and importantly non-biased approach to be made to studying the metabolome. Rather than focussing on certain chemicals, the total biochemical composition can be comprehensively profiled (termed metabolomics) (Fiehn *et al.*, 2001). Another related study, which although subtly distinct is often bracketed under the field of metabolomics, is metabolic fingerprinting. This involves the use of the metabolome to provide a rapid fingerprint from which to compare samples such as in identifying strains of bacteria (Goodacre *et al.*, 1996). Confusingly, some toxicological studies refer to metabolic fingerprinting as 'metabonomics' (Kenney & Shockor, 2003); this has led to many authors, notably Sumner *et al.* (2003), to request a standardisation in terminology.

A key development in metabolomics (in the sense that it simultaneously analyses the full complement of metabolites in a sample) has been the increased availability of suitable technology. There are many technologies accessible to a metabolomics user with the choice depending on the balance between speed, selectivity and sensitivity (Sumner *et al.*, 2003). Mass spectroscopy has been the most commonly used (Goodacre *et al.*, 2002) and has proven to be a powerful tool when coupled to liquid or gas chromatography (LC-MS and GC-MS respectively). However, many

metabolic fingerprinting studies have favoured Fourier Transform Infrared Spectroscopy (FT-IR) instead of mass spectroscopy. Infrared spectroscopic methods have an advantage over mass spectroscopy in that they are non-destructive and lack reagents (Ellis *et al.*, 2003). The premise of FT-IR is that when samples are interrogated with light, different chemical bonds will absorb particular wavenumbers and vibrate differently (Bauer & Richter, 1996). Thus the absorbencies can be correlated to functional groups on molecules. The mid-infrared region (4000-6000 cm^{-1}) is chemically the most informative and thus used for metabolomic fingerprinting. Furthermore, a key development in the efficacy of FT-IR came when Goodacre *et al.* (1996) pioneered the use of diffuse reflectance-absorbance methods in metabolomic fingerprinting as opposed to the passing of the infrared beam through a substance (where the absorbance was recorded). By plating samples on a sand-blasted aluminium plate and loading this on the motorised stage of a reflectance TLC accessory it became possible to analyse up to 400 samples in one run with each sample taking a matter of seconds to record.

FT-IR has been of great use in microbiological metabolomic studies. There have been numerous studies that have used FT-IR to identify bacterial strains that has obvious biomedical applications. For example, Goodacre *et al.* (1998) used FT-IR, coupled with appropriate chemometric methods, to discriminate between various urinary tract infection causing strains. This alleviates the need for the time-consuming and expensive conventional identification techniques. The same group also used FT-IR to differentiate between *Enterococcus* and *Streptococcus* strains (Goodacre *et al.*, 1996). Winson *et al.* (1997) used similar methods to assess rapid screening of metabolite

overproduction. This technique was originally pioneered by Helm *et al.*, (1991) who realised the great potential of spectroscopy in bacteriology.

With the success of FT-IR in bacterial research, there was a spate of papers using FT-IR in related but nonetheless different fields. Preliminary analysis of the *Saccharomyces cerevisiae* metabolome with FT-IR was seen as a fundamental step in the post-genomic study of the yeast genome (Oliver *et al.*, 1998). One mycological study, which illustrated the potential of FT-IR to ascertain evolutionary relationships, used FT-IR and Py-MS to differentiate between different *Candida* strains (Timmins *et al.*, 1998). FT-IR was also used to rapidly detect the onset of food spoilage in poultry meat samples which showed that FT-IR has potential commercial applications in the food industry (Ellis *et al.*, 2002).

The progression of FT-IR use from prokaryotes to eukaryotes has already extended to more evolutionarily derived organisms, with progress continually underway with plants. Whilst most plant metabolomic studies use mass spectroscopy, research by Johnson *et al.* (2000; 2003; 2004) has shown a diverse range of uses for plant-based studies using FT-IR. This approach could discriminate between two different strains of tomatoes and whether salt-stress affected the metabolome (Johnson *et al.*, 2003). A previous study highlighted the crucial need for appropriate chemometrics in order to make the results meaningful; in this case genetic programming of simple interpretable rules was used (Johnson *et al.*, 2000). This provided potential biomarkers for detecting salt-stress. FT-IR was also used to help optimise red clover ensilage (Johnson *et al.*, 2004). The importance of this work is in laying solid foundations from which the study of plants via infrared spectroscopic methods can be built.

The current trend in using FT-IR to analyse biological systems of increasing complexity has already been extended to the most complex biological systems of all; namely ecosystems. Studies into how earthworm casts alter due to varying litter types have been shown by FT-IR (Scullion *et al.*, 2003). Related studies by Gwynn Jones *et al.* (2004) showed that UV-B induced alterations in litter could be identified in earthworm casts. Gidman *et al.* (2003) showed that metabolomic fingerprinting could show plant-plant interference in artificial systems where *Arabidopsis thaliana* and *Brachypodium distachyon* were grown. This study is useful in exemplifying the importance of metabolomic fingerprinting as a driving force in hypothesis synthesis as opposed to its use in testing hypotheses. By showing that there is a difference, and perhaps indicating where that difference may lie, FT-IR could help elucidate some of the chemical bases of ecological interactions.

Plant material from all the competition experiments in this thesis were analysed by FT-IR. The alterations to the metabolome were then compared to the morphological alterations to the plant to determine whether the metabolome reflects structural changes. This provided the basis for a technique to ascertain the physical environment of a plant by obtaining simple metabolic fingerprints. After presenting the key hypotheses of this thesis, the following chapters outline the methodological optimisation of both metabolic fingerprinting and the systems used to expose the plants to exposed UV-B.

1.6: *Hypotheses*

(1) UV-B could alter the competitive balance by differentially affecting the biomass of different species

(2) UV-B provides a suitable and easily administered abiotic stress from which to test the theories of Tilman and Grime

(3) Soil leachates could reflect changes to aboveground communities and UV-B

(4) Metabolic fingerprinting by FT-IR could detect alterations to the metabolome induced by competition and UV-B

Chapter Two: Development of a Supplemental UV-B System

2. 1: Introduction

All the experiments in this thesis use UV-B radiation (280-320 nm) as a technique to induce stress in plant interactions. The central reason for its use is that it is an effective stress-causing agent, commonly known to reduce biomass, which can be accurately administered to study the effects of stress on plant biomass (Newsham *et al.*, 1996). In addition to this role, UV-B is also highly ecologically relevant as concerns of ozone depletion have suggested an increase in ambient UV-B (Björn *et al.*, 1997). Furthermore, revisionist studies suggest that it is one of the most ubiquitous plant stresses and that it may have a great impact on natural communities regardless of ozone depletion (Paul, 2001; Paul & Gwynn Jones, 2003). The following introduction outlines the principal considerations in experimental design which have often caused considerable disagreement amongst researchers. Results from the optimisation of the three enhanced UV-B facilities used throughout this project are then presented and discussed for their suitability.

The first consideration when starting a UV-B experiment is the location. There have been numerous studies based in glasshouses (van de Staaij *et al.*, 1997), controlled growth cabinets (Döhring *et al.*, 1996) and outdoor field sites which irradiate either empty plots (Aphalo *et al.*, 1999; Holmes *et al.*, 2002) or natural ecosystems (Björn *et al.*, 1997). The choice of system is fundamentally based on the hypotheses tested. If the effects of UV-B as a stress are required then glasshouses and growth cabinets have the advantage that all variables can be controlled and levels of UV-B can be

extremely high. Thiel *et al.* (1996) developed a phytotron with the potential extraterrestrial UV-B strength of 30 W/m^2. A drawback of such systems is that it is hard to extrapolate data from controlled conditions to natural systems (McLeod, 1997). Therefore, if the effects of UV-B on natural communities are required then UV-B frames which irradiate natural communities are more suitable.

Another initial consideration is whether the timing of the lights follows a square wave (SQW) or modulated pattern. SQW designs are the most simple whereby a series of lights are switched on at full power for a certain number of hours and the UV-B dose in $KJ/m^{-2}/day^{-1}$ can be calculated. Modulated designs employ sensors that monitor the ambient UV-B and adjust the power of the lamps so that the percentage increase of UV-B is always constant. This is especially useful as UV-B varies significantly due to cloud cover and throughout the day and year. Whilst modulated designs are more complex, they remain widely used and some of the first systems adopted this approach (Caldwell *et al.*, 1983; Newsham *et al.*, 1996; McLeod, 1997 & Aphalo *et al.*, 1999). Sullivan *et al.* (1994; cited in McLeod, 1997) carried out a study that compared the two designs and found that the communities responded differently to similar doses of UV-B from a SQW design to a modulated design. A similar experiment conducted by Musil *et al.* (2002) found that differences occurred although concluded that SQW designs were still ecologically relevant. The choice mainly depends on economic limitations (modulated designs are considerably more expensive) but also the hypotheses tested. A modulated design is the most suitable for assessing the effects of a known level of ozone depletion although SQW are the most convenient for assessing the extent of UV-B as a stress and for administering precise quantities of UV-B.

The use of appropriate controls has also become one of the key issues in UV-B experimental design. The majority of the earlier field studies utilised elevated UV-B frames and control frames only. One of the major disadvantages of such a design is that the UV frames possess both elevated UV-B and UV-A. Whilst the amount of UV-A emitted from the lamps represents a small fraction of ambient UV-A (Petropoulou *et al.*, 1995) it has been shown that the UV-A released from the lamps elicits significant biological effects. Newsham *et al.* (1996) incorporated UV-A controls and found altered height and herbivory in *Quercus robur* in both UV-B and UV-A control frames. Caldwell *et al.* (1994) also found that UV-B only affected plants if the levels of UV-A and PAR were below half of the sunlight flux. Even though UV-A controls have been shown to have no effect (Niemi *et al.*, 2002) and Newsham *et al.* (1996) suggested that the similar effects could be due to general properties of fluorescent lamps, it is now widely accepted that UV-A controls are advised in such research.

Given the importance of UV-A controls there has been much debate into the best filters to use. Filters are commonly used to remove high energy UV-C which is present in the lamps but not in the atmosphere. However, filters are also needed for the UV-A controls which cut out wavelengths at around 320 nm (removing UV-B but allowing UV-A through). The most common option is to use cellulose acetate (CA) to attenuate UV-C and a polyester filter (usually Mylar) to remove UV-B. Döhring *et al.* (1996) noted that CA requires frequent replacement and opted for borosilicate glass filters instead. Holmes *et al.* (2002) engineered a polychromatic irradiation system which used plastic and glass filters instead of CA and Mylar respectively.

These had the further advantage of being mechanically stable and did not degrade as quickly. Tevini *et al.* (1990; cited in McLeod, 1997) used an ozonised air layer to attenuate UV-C in a growth cabinet although this has even greater safety implications and is difficult to maintain. It is therefore recommended that a variety of filter combinations are carried out prior to a major study to ensure optimum filtration.

The University of Wales, Aberystwyth possesses three UV-B facilities that allow for numerous hypotheses to be tested. The first is a small frame based in a glasshouse for small scale experiments using low levels of UV-B, the second is a growth cabinet allowing for natural levels of UV-B to be studied under controlled conditions and the third is an outdoor field site based at Penglais farm whereby elevated UV-B can be studied. The following sections will discuss the set-up of these designs and present data from spectroradiometry analysing the strength of the radiation. The results will then be discussed in the context of similar studies.

2.1.1: *Aims*

1. Take spectral measurements of the three facilities used to expose plants to UV-B: Namely the UV-B field site, growth cabinet and glasshouse frame
2. Take spectral measurements of numerous lamp coverings to ensure optimum UV-B and UV-A exposure

2.2: Materials & Methods

2.2.1: *Measurement of Ultraviolet Radiation*

All UV-B spectra were collected using a portable EPP2000 Fibre Optic Spectrometer with a F400 UV/VIS fibre optic cable (StellarNet Inc, Tampa, Florida, USA). The EPP2000 was connected to a laptop (running under Windows XP) via an USB2 interface using SpectraWiz software (StellarNet Inc, Tampa, Florida, USA). The EPP2000 was powered by a 5V power supply and could collect between 200 nm and 850 nm at a resolution of 0.5 nm. Biological weightings ($UV\text{-}B_{BE}$) were carried out according to Caldwell *et al.* (1986).

2.2.2: *Field Site Set-Up*

All outdoor experiments were conducted at the Trophic Interaction Field Site at Penglais Farm, Aberystwyth (52.25º N, 04.05 º W) (see Chapters Four and Five). Figure 2.1 shows a diagrammatic representation of the site and location of the frames.

24 frames were arranged into eight blocks of 3 frames allowing a maximum replicate number of n = 8 (see Figure 2.1 for location of the blocks). Each block possessed an UV-A, UV-B and an 'effigy' frame (control – C on Figure 2.1) which had no lamps present although allowed a similar degree of shading to plants as the active frames.

The frames were enclosed in a compound surrounded by a 4 m high barbed wire fence. The frames were surrounded by a gravel pathway with frames in a demarcated

working area (as shown on Figure 2.1). A separate control building opposite the compound controlled the timing of lights and acted as a laboratory allowing for direct research on site.

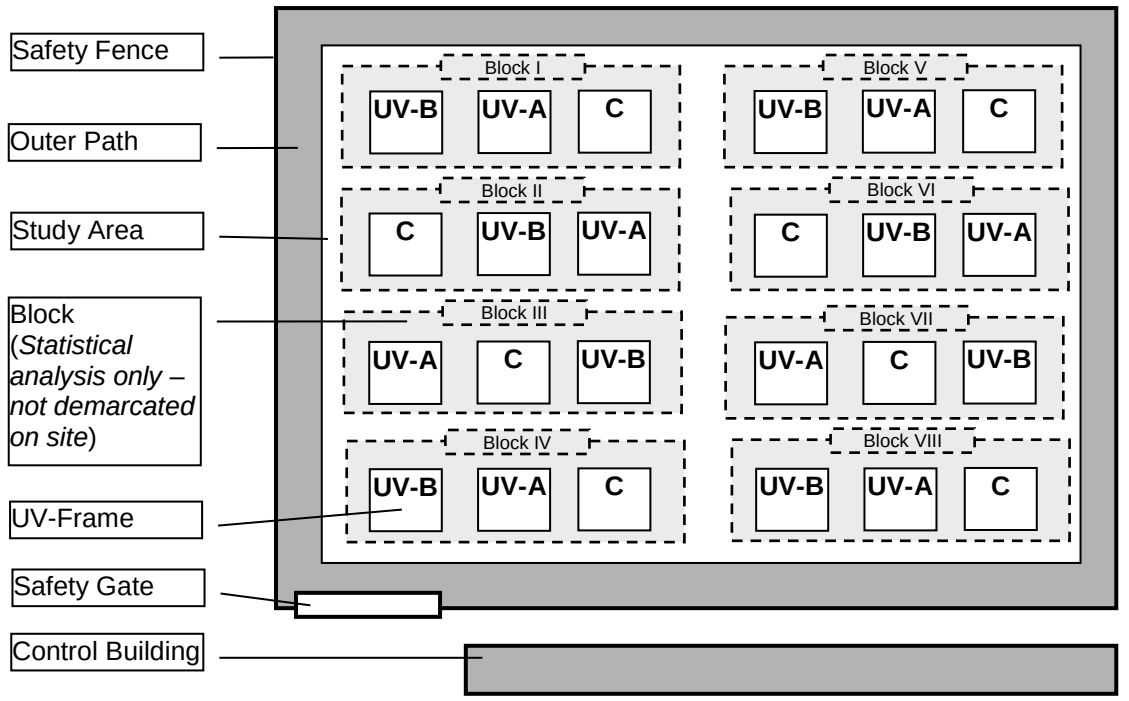


Figure 2.1. Schematic diagram of the Trophic Interaction Field Site at Penglais Farm, Aberystwyth. The frames are grouped into blocks for statistical analysis (shown by dotted line) although the blocks are not demarcated as such on the actual site.

Each individual frame was constructed from aluminium and comprised of two triangular supports linked on either side by two triangular lamp shades also constructed of aluminium to allow all the light to be directed to the ground. This allowed for an area of 1.5 m by 1.5 m to be fully irradiated at a distance of 1 m. Each lamp shade possessed two UV-B lamps (therefore four lamps in total per frame). Q-Panel 313 lamps (Q-Panel, Cleveland, Ohio, USA) were used in both UV-B and UV-A frames. A variety of filters were used to attenuate UV-C (cellulose diacetate,

transparent plastic) and UV-B for the UV-A controls (soda-lime glass, Mylar). The optimal filter was tested and results shown in next section.

2.2.3: *Growth Cabinet*

The two plant growth cabinets were custom-designed from Vindon Scientific (Oldham, Lancs, UK) with a usable growing area of 2 m x 1m with a distance of 1 m from lights. One cabinet acted as a UV-A control and the other as enhanced UV-B. Both frames possessed 400 W sodium lamps (for PAR) and 250 W UV-B lamps. The UV-B cabinet filtered UV-C by using WG-305 glass filters (Schott, Stafford, UK) whilst the control cabinet used Borofloat-33 glass (Schott, Stafford, UK) to attenuate UV-B and allow UV-A.

2.2.4: *UV-B Frame*

A simple rectangular aluminium frame (1m x 2m x 1m) was constructed with two Q-Panel 313 lamps (Q-Panel, Cleveland, Ohio, USA) wrapped in cellulose diacetate (to attenuate UV-C) on the longest two sides. A similar frame lacking the lamps was used as a control. The base was adjustable to alter distance between lamps and plants.

2.2.5: *Abisko Research Station*

The aforementioned experimental designs were developed during the course of this project to test the hypotheses that UV-B stress alters plant competition and metabolic profile. However, Chapter Eight presents an experiment carried out to investigate the

effects of enhanced UV-B on a sub-Arctic heath at the Abisko Research Station in northern Sweden. The UV-B field site has been in operation since 1991 and the design has been optimised and will therefore not be discussed here. Details of the experimental design are therefore mentioned at the start of the relevant chapter.

2. 3: Results

2.3.1: *Field Site*

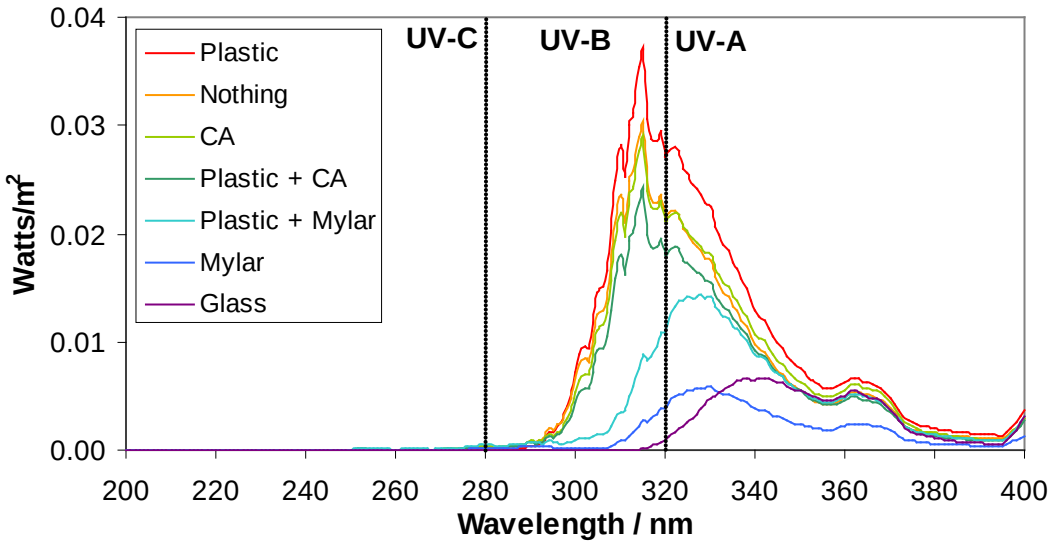


Figure 2.2. Comparison of spectral irradiance of seven types of filtration at the Penglais Farm, UV-B Facility.

The results from the comparison of filters (Figure 2.2) show that CA was not necessary. Levels of UV-C were below 0.01 W/m^2 under the plastic covering even when CA filters were not used (data not shown). Furthermore, similar levels of UV-C were detected under the control frames indicating that the 0.01 W/m^2 was probably due to electrical interference. CA also had the disadvantage of reducing levels of UV-

B. There was a 34.5 % decrease in UV-B between CA-covered lamps and the plastic covering. CA was therefore not used for these reasons.

The plastic coverings also possessed the advantage that levels of UV-B were marginally higher (2 %) than when no covering was used (Figure 2.2). This was corroborated by further experimentation (data not shown) although to an even greater extent. Whilst it is unknown why there is an increase in UV-B, it is possible that the plastic covering could be showing fluorescence. However, the spectra for the two treatments possess a similar shape (Figure 2.2) and this would not be expected if fluorescence was occurring. The optical properties of the plastic may also be directing the light directly downwards rather than diffusing away from the sensors. Given that the plastic covering was also more durable than CA, it was decided that it should be retained.

Table 2.1. Comparison of various UV parameters ($KJ/m^2/hr^{-1}$) for the three UV facilities and ambient UV at the field site at 12:30, 05.06.06.

	UV-A	UV-B	UV-B_{BE}	A:B
Field Site (+UV)	5.38 ± 0.32	3.68 ± 0.20	0.76 ± 0.06	1.46 ± 0.01
Field Site (-UV)	2.18 ± 0.10	0.02 ± 0.00	0.06 ± 0.04	109.0 ± 12.8
Cabinet (+UV)	151.3 ± 5.20	8.30 ± 0.32	5.44 ± 0.24	18.23 ± 1.37
Cabinet (-UV)	20.1 ± 13.3	0.40 ± 0.84	0.48 ± 0.22	50.3 ± 3.53
Frame	2.58 ± 0.02	1.88 ± 0.02	0.34 ± 0.00	1.70 ± 0.01
Outdoor (June)	127.9 ± 0.90	10.0 ± 0.08	7.08 ± 0.12	12.83 ± 0.15

The UV-B frames were found to supplement ambient radiation with 3.68 $KJ/m^2/hr^{-1}$ UV-B and 0.76 $KJ/m^2/hr^{-1}$ UV-B_{BE} (Table 2.1). The UV-B:UV-B_{BE} ratio was 4.8 whilst the ratio under natural conditions at 12:30 on 05.06.06 was 1.4. The decrease

in UV-B_{BE} is likely to stem from the lack of strong radiation between 280 and 300 nm where most of the weighting occurs. This can be clearly seen in Figure 2.3c where there is far less high energy UV-B compared to the natural light shown in Figure 2.3a.

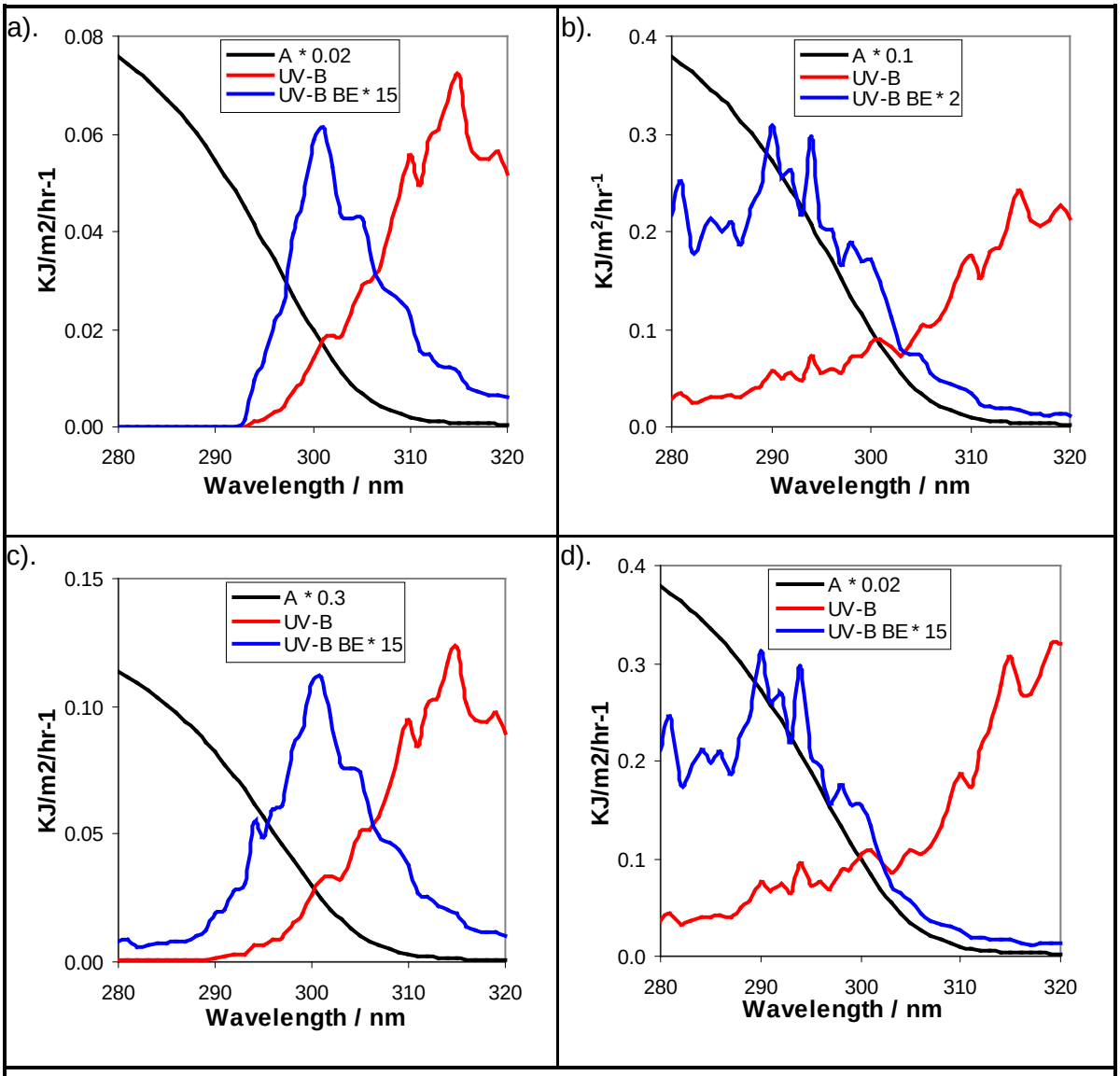


Figure 2.3. Spectral irradiance (red line), biological weighting function (A; black line) and biologically weighted spectral irradiance (blue line) for the UV-B range (280-320 nm) at four different locations: a). UV-B frame; b). growth cabinet; c). field site and d). Natural light on clear day (12:30; 05.06.06).

The three treatments, which were added to remove UV-B but allow UV-A, pose an important problem. All three treatments showed a marked decrease in UV-A

compared to the levels of UV-A generated by the UV-B treatments. There was a 38.7 %, 59.5 % and 72.1 % decrease in UV-A for plastic with Mylar, glass and Mylar respectively. This can clearly be observed by comparing the spectra in the UV-A range of Figure 2.2. Furthermore, both Mylar and plastic with Mylar, allowed a considerable amount of UV-B through: respectively representing 6.5 % and 19.5 % of the UV-B under the UV-B frames. It was therefore not possible, given the available technology, to have a true UV-A control. The UV-A frames effectively represented a low UV-A treatment whilst the UV-B frames represent a high UV-B and UV-A treatment.

The results also show that there is a considerable location effect within the frames. UV levels were significantly higher ($F=3.59$; $P=0.031$) directly beneath the lamps as opposed to the gap between the two lamp-holders which showed a 43.5% decrease in UV-B (data not shown). The variation caused by these differences would therefore confound any biological effects. One solution would be to increase the distance between the plants and the lamps allowing for more uniform coverage of light, although this would compromise the strength of the UV-B. Arranging the plants parallel to the lamp holders would also solve the problem as there was no difference in UV-B running along the length of the lamps ($F=0.37$; $P=0.690$). It was therefore decided to arrange the plants directly underneath the lamps.

Analysis suggested there was no difference in spectral output between the eight blocks ($F=1.77$; $P=0.115$) and more importantly there was no interaction between the radiation treatments ($F=1.27$; $P=0.251$). Another advantage was that any diurnal

differences would be confounded in the blocks as harvesting was completed one block at a time.

2.3.2: *Growth Cabinets*

Results from the quantum sensor showed that the PAR readings in both cabinets were not statistically different (UV-B cabinet: 685 ± 18.4 μmol/m^2/s^{-1} & Control Cabinet: 648 ± 20.0 μmol/m^2/s^{-1}; data for ten readings throughout the cabinet). Levels of UV-B in the treatment cabinet were 8.3 KJ/m^2/hr^{-1}. The ratio of UV-B to UV-B_{BE} was 1.5 which is similar to the ambient ratio in June at 1.4 (Table 2.1). This can be seen in Figure 2.3b (cabinet spectra) which is very similar to Figure 2.3d (ambient spectra in June). The coverage of light was uniform throughout the cabinets (F=23.99; P=0.061). One drawback of the system is that the levels of UV-A in the treatment cabinet were substantially higher than the control cabinet although the levels were comparable to outside measurements (Table 2.1).

2. 3.3: *UV-B Glasshouse Frame*

The UV-B frame emitted 1.88 KJ/m^2/hr^{-1} and 0.34 KJ/m^2/hr^{-1} UV-B_{BE}. The ratio of UV-B to UV-B_{BE} is 5.5 which is more akin to the value previously noted for the field site (4.8) which is unsurprising given the use of the same lamps. The similarities can be noted by comparing Figure 2.3a (glasshouse frame) with Figure 2.3c (field site) with the lower irradiance due to the CA covering of the lamps. The CA covering may also account for the sharp cut-off at 292 nm (Figure 2.3a).

2.4: Discussion

2.4.1: *The Role of Cellulose Acetate*

One of the most surprising conclusions drawn from the results was that cellulose acetate (CA) filters were unnecessary at the field site. UV-C, the attenuation of which is the prime reason for CA, was not detected under the frames. The 0.01 W/m^2 that was detected (Table 2.1; seen on Figure 2.2) was also found under control conditions in the dark suggesting that this was simply electrical interference. Given that CA reduces UV-B by 34.5 % (Table 2.1), there are clear benefits to removing CA filters. This is contrary to the vast majority of studies in natural ecosystems such as sub-Arctic heaths (Bjerke *et al.*, 2003; Gwynn Jones *et al.*, 1997), dune systems (Rozema, 1999), fens (Searles *et al.*, 1999) and Mediterranean scrubs (Musil & Wand, 1999; Manetas, 1999), where use of CA was ubiquitous.

However, it is possible that the widespread use of CA in such experiments was simply following a few original precedents and, given spectroradiometers are not always available, CA is a sensible precaution in case UV-C is present. Furthermore, it is clear that the wavelength quality depends on the type of lamps and plastic coverings used (Figure 2.2), so one can experience a degree of variation between different studies. In support of this, there have been studies that have forsaken CA after extensive spectroradiometric measurements (Holmes *et al.*, 2002; Döhring *et al.*, 1996). Döhring *et al.* (1996) opted for Borosilicate glass which was used in the growth-cabinet design. A drawback of Borosilicate glass is that it is expensive and would incur substantial costs in a large-scale outdoor experiment. This cost could be

offset by the cost (manual and material) of continual bi-weekly replacement of CA filters. Moreover, there is evidence to suggest that CA is phytotoxic to plants (Krizek & Mirecki, 2004). This exemplifies the importance of a thorough study of experimental design before undertaking an enhanced UV-B experiment and also stresses that many studies may not be comparable due to the variation in design.

Another interesting phenomenon was that the plastic covering subtending the UV-B lamps, originally used to hold the CA in place, enhanced the levels of UV-B (Figure 2.2). This was repeatedly tested to confirm its occurrence. As hypothesised in the results section, this could be due to redirection of the light directly beneath the frame or fluorescence. UV-fluorescent plastics are widely available in commercial applications (such as discothèque hoardings) and UV-reflective plastic moulds are even used as an insect-deterrent for glasshouse crops (Summers *et al.* 2004; Summers & Stapleton, 2002) which shows that it is not uncommon for some plastics, and therefore potentially the plastic used in this experiment, to alter the properties of UV-B. The further experimentation of plastic coverings may even prove a suitable, or desirable, alternative to CA.

2.4.2: *Biological Weightings*

Another problem noted from the results was that UV-lamps alter the ambient ratio of UV-B to UV-B_{BE}. The lamps effectively have less biologically effective radiation as a proportion of their spectral output as can be found in sunlight (Table 2.1). For example, at midday on a clear day in July at the field-site, the spectral output is akin to Mumbai although biologically akin to Belgrade (as calculated from Björn &

Murphy, 1985). The same problem applies to the simple UV-B frame (Table 2.1) which is unsurprising given the lamps are the same. The UV-cabinets had no such problems (Table 2.1) and recreate the balance between UV-B and UV-B_{BE} on a clear day in June in Aberystwyth with the added advantage that other environmental variables can be controlled.

However, there has been a large degree of controversy surrounding biological weightings such as the widely used original generalised plant action spectra by Caldwell (1971) and its subsequent modification (Caldwell *et al.*, 1986). There have been numerous studies that suggest generalised action spectra are too broad and a more species-specific approach should be adopted (Quaite *et al.*, 1992; Cooley *et al.*, 2000; Yao *et al.*, 2006a). The controversy was even reappraised by Caldwell & Flint (1997) who suggested that UV-A should be included into the action spectra although this still precludes the issues surrounding the validity of generalised spectra. Therefore, it is likely that such weightings for a *Lolium-Lotus* system may need to be optimised on an individual basis and it could even differ for the two-species. It is suggested that for future work, given economic and technological constraints, action spectra specific to the component species are calculated.

2.4.3: *UV-A Frames*

A significant problem with UV-A controls was also apparent from this study. UV-A levels were clearly higher under UV-B lamps than under the actual UV-A controls to the extent that the UV-A frames cannot truly be called controls. Figure 2.2 shows that glass attenuates UV-A by 59.5 % and Mylar by 72.1 % despite both being lauded as

ways to attenuate UV-B although keeping UV-B the same. However, in both cases, the supplemental UV-A is far smaller than that of sunlight (5.28 W/m^2 under glass compared to 128 W/m^2). This has also been pointed out by Petropoulou *et al.* (1995). The problem was even more exacerbated between the growth cabinets (Table 2.1).

Despite investment into UV-B field sites continuing (Boelen *et al.*, 2006), it is clear that the technology to create a perfect UV-A control is not yet available (Krizek, 2004). White & Jahnke (2002) also concluded that a system based on CA and Mylar was not appropriate after an experiment using a potassium chromate (K_2CrO_4) liquid filter to attenuate UV-B on an algal study. However, whilst liquid filters may be suitable for phycology there are clear drawbacks in a large-scale outdoor study. This study has opted to use glass given it was the best option available. More research into types of filters is therefore imperative given UV-A controls are of great use.

2.4.4: *Conclusion*

In conclusion, it is clear that research into UV-B is hampered by many problems in experimental design. The options available are far from satisfactory and the principal decision facing a researcher is in maximising the suitability given the constraints. In this case, it was clear that UV-A controls posed a substantial problem that was minimised by using glass. In the growth cabinets the problem was even greater although the problem of replicating sunlight was solved by using Borosilicate glass. Given these caveats, the set-up presented provides a suitable basis for plant research.

2.4.1: *Summary*

1. Cellulose acetate was not needed at the field site which exemplifies the fact that each enhanced UV-B facility necessarily requires specific parameters which hinders meta-analysis of international sites
2. It was impossible to create a true UV-A control at the field site although UV-A frames were included to assess whether the effects of enhanced UV-B and low exposures of UV-A were comparable
3. Despite the difficulties faced in experimental set-up, all three facilities can be used to assess the effects of supplemental UV-B

Chapter Three: Development of a Metabolic Fingerprinting System

3.1: Introduction

This section presents the methodology for the FT-IR technique that will be used throughout the project to ascertain the effects of stress on competitive interactions. An experiment was set-up that investigated the effects of UV-B on the biomass of *Lolium perenne* and *Lotus corniculatus* var. *japonicus*. Metabolic samples from a variety of growth media and organs were taken and stored under a variety of conditions. The results are presented and consideration is given to which methodologies are most suitable for this project.

3.1.1: *Aims*

1. Optimise a methodology for obtaining metabolic fingerprints of plant samples using FT-IR
2. Determine whether different techniques for storing plant samples have an effect on the metabolome
3. Assess whether supplemental UV-B from a glasshouse frame affects the metabolome of plant samples

3.2: Materials & Methods

The following materials and methods section is divided into three sub-sections. The first sub-section describes the methodology used to take metabolic fingerprints. Two instruments were used; the first (Bruker IFS28) used the reflectance absorbance technique whereby a plant sample was put onto a metal plate so the infra-red beam could literally pass through the sample, 'hit' the metal plate, and then be reflected on to the detector. The second instrument (Bruker Vertex 70) used the transmission technique whereby the sample was loaded onto an opaque silicon plate so the infra-red beam passed through both the sample and loading plate onto the detector. The Vertex 70 was procured towards the end of the project as a replacement to the IFS28.

The second sub-section describes the two types of data analysis that were used. Both employ Principal Component Analysis (PCA) dimension reduction at the start. The first technique uses scatter plots to visualise patterns. The second analysis uses ANOVA or MANOVA to determine any patterns.

The third sub-section describes an experiment that was carried out to determine which types of storage are best suited to plant materials and offers an opportunity to optimise the technique for future analysis. The optimisation of the technique is given in the results section.

3.2.1: *FT-IR Methodology*

3.2.1.1: IFS28 Protocol

The Bruker IFS28 FT-IR Spectrometer (Bruker Spectrospin Ltd., Banner Lane, Coventry, UK) was equipped with an MCT (mercury-cadmium-telluride) detector cooled with liquid N_2. The following protocol using the IFS28 has been used extensively in FT-IR studies (e.g. Ellis *et al.*, 2002; Goodacre *et al.*, 1996, 1998, 2002; Timmins *et al.*, 1998). Aliquots of 5 μl were evenly applied onto one of 400 wells drilled onto a sandblasted aluminium plate (measuring 10 cm by 10 cm). The samples were arranged randomly to minimise the effects of artifactual trends in the data (e.g., edge effects) (Johnson *et al.*, 2003) and oven-dried at 50ºC for one hour prior to analysis. The IFS28 was under the control of an IBM-compatible personal computer using OPUS 2.1 software running under the IBM OS/2 Warp operating system (provided by manufacturers). Spectra were collected over the wavenumber range 4000 cm^{-1} to 600 cm^{-1} at a resolution of approximately 3.85 cm^{-1} and acquired at a rate of 20 s^{-1}. There were therefore 883 variables per sample. To improve signal-to-noise ratio, 256 spectra were co-added and averaged. Spectra were displayed in terms of absorbance as calculated from the reflectance-absorbance spectra using the Opus software (based on the Kubelka-Munk theory) (Gidman *et al.*, 2003).

3.2.1.2: Vertex 70 Protocol

The Bruker Vertex 70 Spectrometer (Bruker Spectrospin Ltd., Banner Lane, Coventry, UK) measures transmission as opposed to reflectance and therefore does not need to

be cooled with liquid N_2. Samples were prepared as with the IFS28 and plated onto re-usable silicon plates. These were oven-dried at 50ºC for 30 minutes prior to analysis. The Vertex 70 was controlled by an IBM-compatible personal computer using OPUS version 5.5 (provided by manufacturers) under the Windows XP operating system. Spectra were collected from 4000 cm-1 to 400 cm-1 at a resolution of 4 cm-1 (1674 variables) with 32 scans per sample. The aperture of the beam was set to 3mm which was sufficient to enable readings to occur whilst reducing excessive absorbance from the transmission technique. As with the IFS28, spectra were displayed in terms of absorbance.

3.2.2: *Data Analysis*

3.2.2.1: Dimension Reduction

ASCII data were exported from the Opus software used to control the IFS28 and imported into Matlab version 6.0 (The MathWorks, Inc., 24 Prime Par Way, Natick, MA, USA), which runs under Microsoft Windows 98/XP on an IBM-compatible personal computer. The CO_2 band was then removed as it is common to all samples.

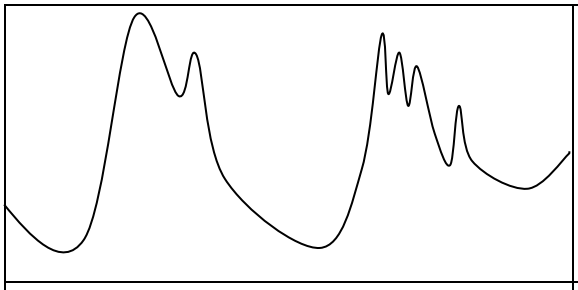
PCA (principal components analysis) was initially performed on the data. PCA reduces the dimensionality of the data set whilst retaining as much of the original variance as possible. This is achieved by transforming a number of potentially correlated variables into a smaller number of uncorrelated variables termed principal components; the first principal component possessing the highest variance. Typically,

the first ten principal components explained 99.99% of the original variance in all experiments carried out, thus illustrating the power of this technique.

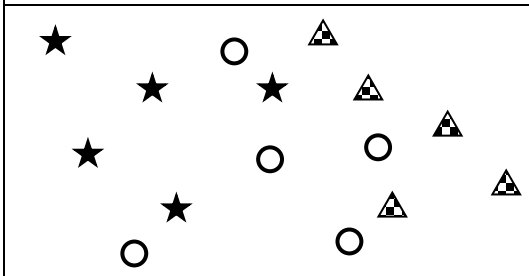
3.2.2.2: Scatter Plot Analysis

By producing a scatter diagram of principal component scores for the first and second principal components it is therefore possible to gain a two-dimensional view of the data which represents the closest distribution of the points in their original hyperdimensional space. The closer the points are on the chart, the more related they are thus allowing clustering analysis to determine similarities (Figure 3.1).

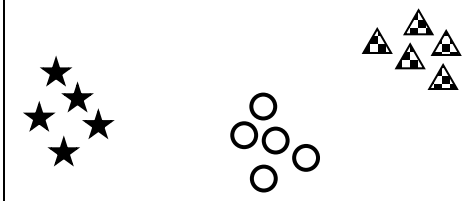
However, in many metabolomic fingerprint samples there will be great similarities in the spectra and to some extent this is to be expected. For example, UV-B may alter the chemical composition of leaf phenolics but the vast majority of chemicals that comprise the structure and function of the leaf will be shared by both (e.g. chlorophyll and cellulose). This results in PCA plots that show no specific clustering. Such is the similarity in chemical structure of plant organs that in some experiments PCA could not sufficiently distinguish between a leaf and a root. As a result of this, supervised clustering methods such as DFA (discriminant function analysis) were used. This required *a priori* knowledge of the data set and aimed to maximise between-group variance whilst minimising within-group variance. As it is not possible to use collinear variables or too many variables in DFA, the inverse of the pooled variance-covariance matrix (from PCA) had to be used, thus making PCA a crucial first step in data analysis.



The 882 wavenumbers per sample in FT-IR analysis require multivariate analysis



PCA converts the original data set into a set of uncorrelated principal components that nonetheless retain the majority of the original variance



DFA uses *a priori* information from the PCA to minimise within group variance and maximise between group variance. Cross validation is used for robustness.

Figure 3.1. Flowchart of methods used to analyse FT-IR data (adapted from Goodacre *et al.*, 1998).

As the results from the DFA may be unreliable it is necessary to use cross validation to assess the robustness of the data (called PC-DFA or cross validation). This involves dividing groups within a FT-IR data set into test sets and training sets. The training set (usually two-thirds of the data set) forms a model upon which previously unseen data (the test set) is projected. Should the test set fall within the clusters formed by the training set then the DFA model is validated and conclusions can be reliably made. It is of utmost importance that machine replicates are not divided, as this would only validate the reliability of the replicates and not the model formed by the groups. The optimum number of principal components used in a DFA model is also determined by cross validation as the program allows many diagrams using different principal components to be made. If too many principal components are

used then the training set becomes tightly clustered and the test set falls out of the training cluster remit (over-trained), conversely, if too few principal components are used then there will not be enough data for the training set to be based upon.

3.2.2.3: ANOVA Analysis

An alternative to using PC-DFA is to employ ANOVA or MANOVA. PCA is initially conducted on the data matrix to reduce dimensionality. The number of principal components that account for 95 % of the variance are then extracted. This is typically between just one and five for most researchers in this field (Gidman, pers. comm.). Should just one principal component account for more than 95 % of the variance then the 882 or 1673 variables from the PC-DFA have been effectively summarised in just one number. This can then be analysed by ANOVA to see whether there were any treatment effects. Should more than two principal components account for more than 95 % of the variance then these can be analysed by MANOVA. The resulting data can be visualised using canonical variant analysis (CVA), which uses different sized circles to show variability and similarities between treatments. Any overlap indicates a lack of differences. An advantage of using this technique is that it gives a probability value for whether there are differences between data and, for ANOVA at least, there is no confusion trying to visualise differences using scatter plots.

3.2.3: *Storage Experiment*

3.2.3.1: Plant Material & Set-Up

Seeds of *Lotus japonicus* 'Gifu' were germinated and grown in 10cm diameter plastic pots for 16 weeks before transferral to empty syringes (June, 2003) (n=48). The syringes were used as the hole in the bottom could be used to collect the leachate from the plants. Care was taken to ensure that no roots were damaged and no soil remained on the roots. The roots were inserted into a syringe holder (the central receptacle without needle or plunger; hence the term 'syringe lysimeter') and filled with vermiculite until the top demarcation printed on the syringe (60ml allowing for space occupied by roots). The syringe lysimeters were held upright in plastic troughs allowing for free drainage (12 syringes per trough and four troughs in total). Plants were watered twice daily (at 9:00 am and 14:00pm) with Hoagland's solution. After one week to allow for recuperation and resumed growth, half the troughs were placed under the aluminium UV-B frame described in Chapter Two whilst the other half were put under control conditions (no UV-B). Another 24 plants were grown under the frames in darkened one-litre hydroponic bottles filled with Hoagland's solutions (replaced every two days in order to re-oxygenise the solution). This was in order to see whether growth technique (syringe lysimeter and hydroponic bottle) had an effect on the metabolome. After two months, roots, shoots and flowers were harvested for dry-weight biomass with four samples taken from each plant for metabolomic analysis.

3.2.3.2: Storage Techniques

The four 3 cm leaf samples taken from each plant were stored by four different methods. The first involved immediate submersion of a sample in liquid N_2 and storage at -80°C. This will be referred to, in accordance with common parlance, as the 'Fresh' storage option which is a misnomer given a truly fresh sample would not be stored at all. However, given the common use of this term it will be retained herein. The second technique (the 'Freeze-Dry' technique) involved immediate flash-freezing of the sample in liquid-nitrogen before freeze-drying in a freeze-dryer (Freeze Dryer 3.5, Birchover Instruments Ltd., Hitchin, Herts, UK). After freeze-drying the samples were stored at room-temperature. The third technique involved immediate freezing of the sample before storing in a drying-oven at 60°C (the 'Freeze-Baked' technique). The fourth technique involved simply storing the sample at 60°C in a drying-oven prior to analysis.

Root exudates were taken from plants grown in the syringes by plugging the hole with small pieces of doweling and filling the main body of the syringe with 20 ml distilled water (which was sufficient to fill the 50 ml lysimeter given the presence of vermiculite and roots). After three hours, the plug was removed and the remaining fluid was collected in an eppendorf tube (there was typically 1 to 3 ml remaining). These leachates would possess any soluble chemicals released from the roots. A preliminary trial (data not shown) showed that the extracted solution was too dilute to be detected by FT-IR so the samples were concentrated by lyophilising the mixture (Savant AES2000 Automatic Environment SpeedVac with VaporNet, GMI Inc., MN,

USA) until it was dry and rehydrating using 0.1 ml of distilled water. Three monthly harvests were taken starting in mid-June 2003 (three weeks after start).

The samples were analysed after two-weeks using metabolomic fingerprinting. Each sample was put into a 2ml eppendorf tube with a 3mm steel ball-bearing and then ground in a mill for thirty seconds. The resulting fine powder was then mixed with distilled water to form a paste that could be easily pipetted onto the plates for FT-IR analysis An initial dilution-series experiment using dilutions of 10 ml to 100 ml of distilled water to 1 mg of ground material was conducted. Based on the results of this experiment, the other samples (flower, root and shoot) were analysed according to this dilution (see results).

Another preliminary test determined whether single-channel analysis was more effective than absorbance analysis. Single-channel analysis involves running the whole plate without samples before the true run so that the effect of each well can be accounted for. Absorbance is where the plate is not run first and a single reference well is used to ascertain the effect of each well on the individual samples.

3.3: Results

3.3.1: *Optimisation*

3.3.1.1: Single Channel & Absorbance

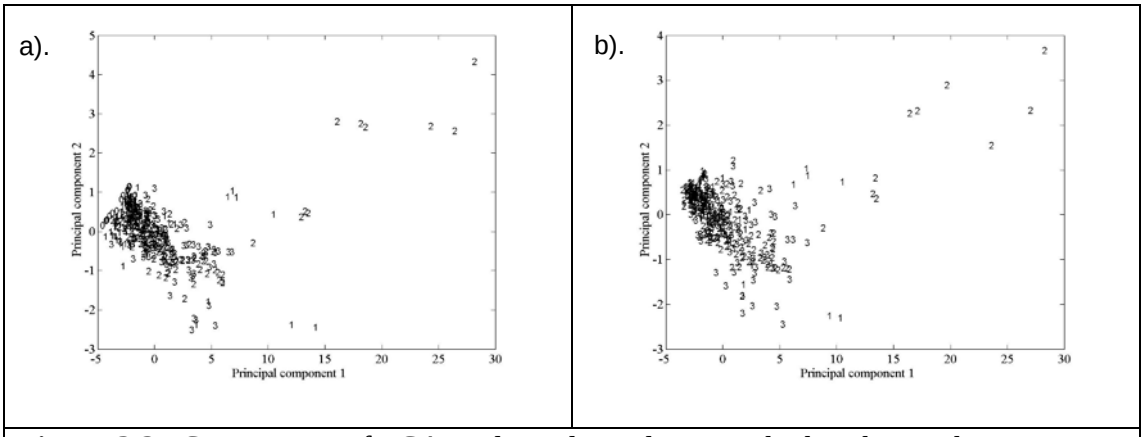


Figure 3.2. Comparison of PCA analysis depending on whether the machine was run under a). single-channel conditions or b). absorbance mode. The example in this case was taken from time of weekly exudate harvest (number denotes week).

The data from Figure 3.2 suggest that there is no significant difference between running FT-IR in single channel or absorbance mode. This can be most clearly seen at the top right of Figures 3.2a and 3.2b where two groups of three replicates (labelled '2') have corresponding positions. This can also be seen toward the bottom of the figure with a pair of figures labelled '1' and a group of numbers labelled '3' being in similar positions on both graphs. These data were taken from root exudates (with the number being week of harvest) although this pattern would occur for any type of material. This shows that the reference well is sufficiently representative of all the wells and there is therefore no advantage to running a blank plate prior to putting the samples on the plate. This saves a considerable amount of time especially given a 400

well plate takes in excess of four hours and on occasions two 400 hundred well plates may need to be run for one experiment. Additionally, this would open the possibility of plating samples in the field which is especially useful if samples are harvested abroad yet the analysis would need to take place in another institution.

3.3.1.2: Spectral Processing

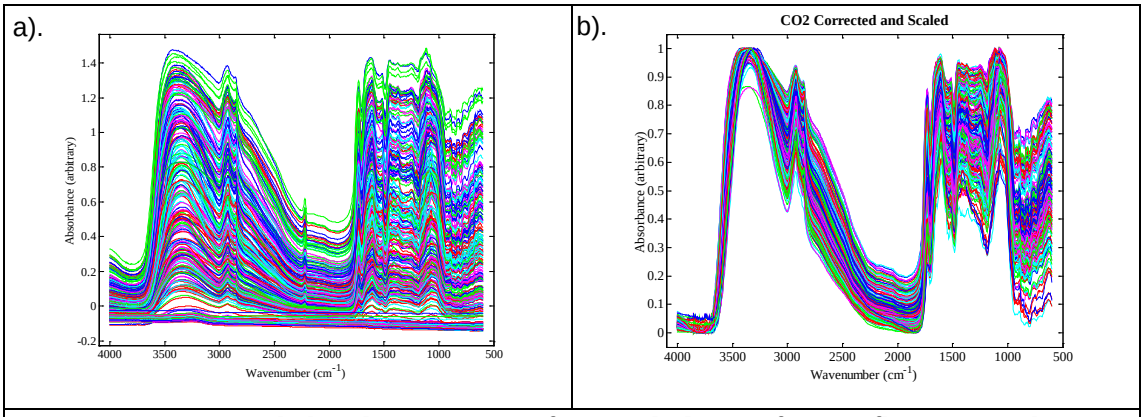


Figure 3.3. Typical absorbance spectra for metabolome of *Lotus* flower; a). shows raw absorbance whilst b). shows absorbance scaled between 1 and 0 with CO_2 band (around 2300 cm^{-1}) removed.

Before statistical analysis, the spectra were processed from their raw form (Figure 3.3a) by removing the CO_2 band and scaling between 1 and 0 (Figure 3.3b). Whilst that is common practice amongst researchers it had no bearing on the results in any experiments (when compared to the raw spectra) although was always conducted as a precaution in case any differences arose.

3.3.1.3: PC-DFA or ANOVA

Figure 3.4 shows the difference between scatter plots and ANOVA. The pattern between the two types of analysis is clear. However, the ANOVA analysis has the advantage that it gives a precise figure for the probability that there is no difference and is more clearly readable. One particular strength of the scatter plot is that there appear to be clear differences for the three dried storage types whilst according to the ANOVA there is statistically no difference between the two. Given the fact that ANOVA or MANOVA give a precise probability value this technique is advantageous. However, a scatter plot will always be plotted first and in those cases throughout the thesis where there is a conflict between the two techniques, both types of graph will be presented.

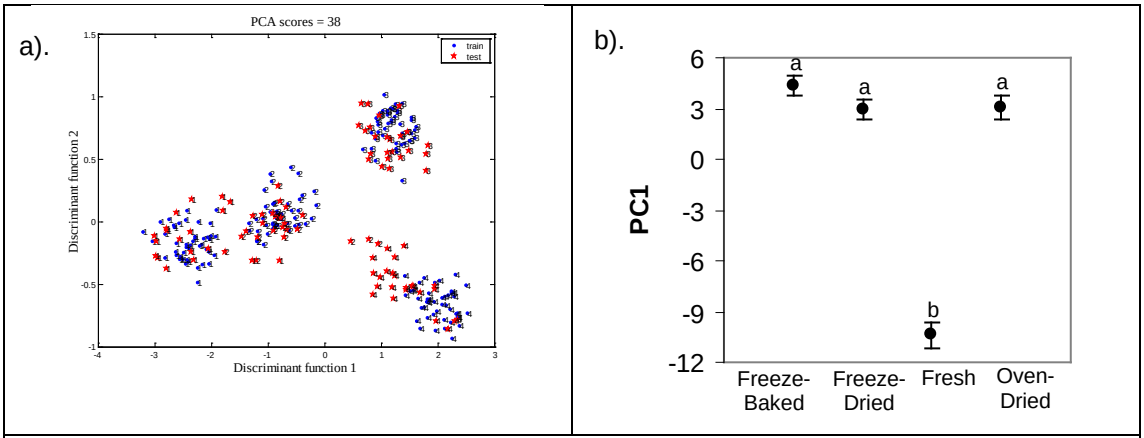


Figure 3.4. Comparison of PC-DFA (cross-validation) and ANOVA analysis. Data taken from the four types of storage in leaves grown in hydroponic bottles (1=Fresh; 2=Freeze dried; 3=Freeze/Oven-Dried; 4=Oven-Dried).

3.3.1.4: Dilution Series

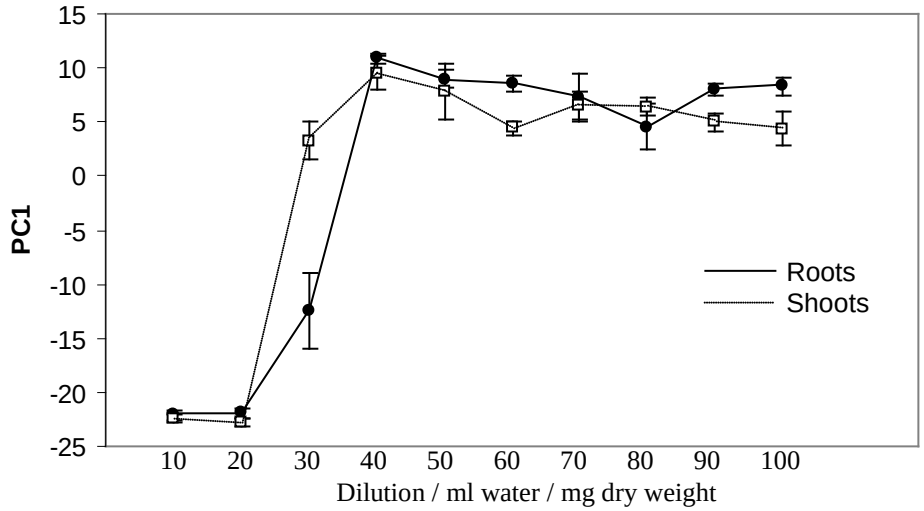


Figure 3.5. Alteration in metabolome with increased dilution for roots and shoots. Dilutions after 40ml water/mg dry material showed no differences.

The dilution series showed that the lowest dilutions (10 and 20 ml water / mg dry material) were statistically similar whilst all dilutions from 40 ml water / mg dry weight were similar (Figure 3.5). Empirical observations suggested that the lowest dilutions made pipetting difficult due to clogging of the tips whilst the highest dilutions made ascertaining whether the wells were filled more difficult. 50 ml water / mg dry weight was therefore chosen as it represented a compromise between the two extremes and was sufficient for a measurement to be recorded.

3.3.2: *Storage Experiment*

3.3.2.1: Biomass

It is clear that there are substantial differences between the two growth techniques of hydroponic bottles and syringe lysimeters (Table 3.1). Under control conditions, the average number of flowers for the hydroponic bottle was 3 whilst it was 23 in the lysimeters. This suggests that hydroponic bottles were detrimentally affecting the plants, which is supported by the 50 % reduction in biomass in the bottles. UV-B did not affect the biomass of the plants although it did increase the flowering number for both hydroponic bottles and syringe lysimeters.

Table 3.1. Table showing the biomasses (g) and flower number for *Lotus japonicus* grown in either lysimeters or hydroponic bottles under UV-B frame or control conditions. No significant differences were present between control and UV-B.

	Hydroponic Bottles		Lysimeters	
	Control	UV-B	Control	UV-B
Flower number	2.75 ± 1.97	3.67 ± 2.21	22.75 ± 4.41	28.71 ± 6.37
Roots	3.66 ± 1.20	2.86 ± 1.09	4.52 ± 1.39	4.10 ± 1.12
Shoots	7.97 ± 1.04	7.94 ± 1.04	10.76 ± 3.14	10.75 ± 2.45
Total	10.80 ± 2.04	11.63 ± 1.86	15.23 ± 4.41	14.86 ± 3.31

3.3.2.2: Metabolic Fingerprinting of Storage Material

For all growth media and organs, fresh material was the most different and also had the largest variance; possibly as the ground material was the least consistent (Figure 3.6). Freeze-drying, oven-drying and freeze-baking were not statistically different for

aboveground organs. Oven-drying differed from the other dried samples in the roots. Given that there was no difference between the dried samples, it is a matter of preference which one is chosen. As organs are frequently dried for biomass analysis, the fact that oven-drying loses no more metabolomically relevant information than freeze-drying is of particular benefit; especially as a direct test between the organ measured and metabolome can be obtained. However, it was noted from empirical observations that freeze-drying formed the finest powder which made it the easiest to pipette. Therefore freeze-drying was used.

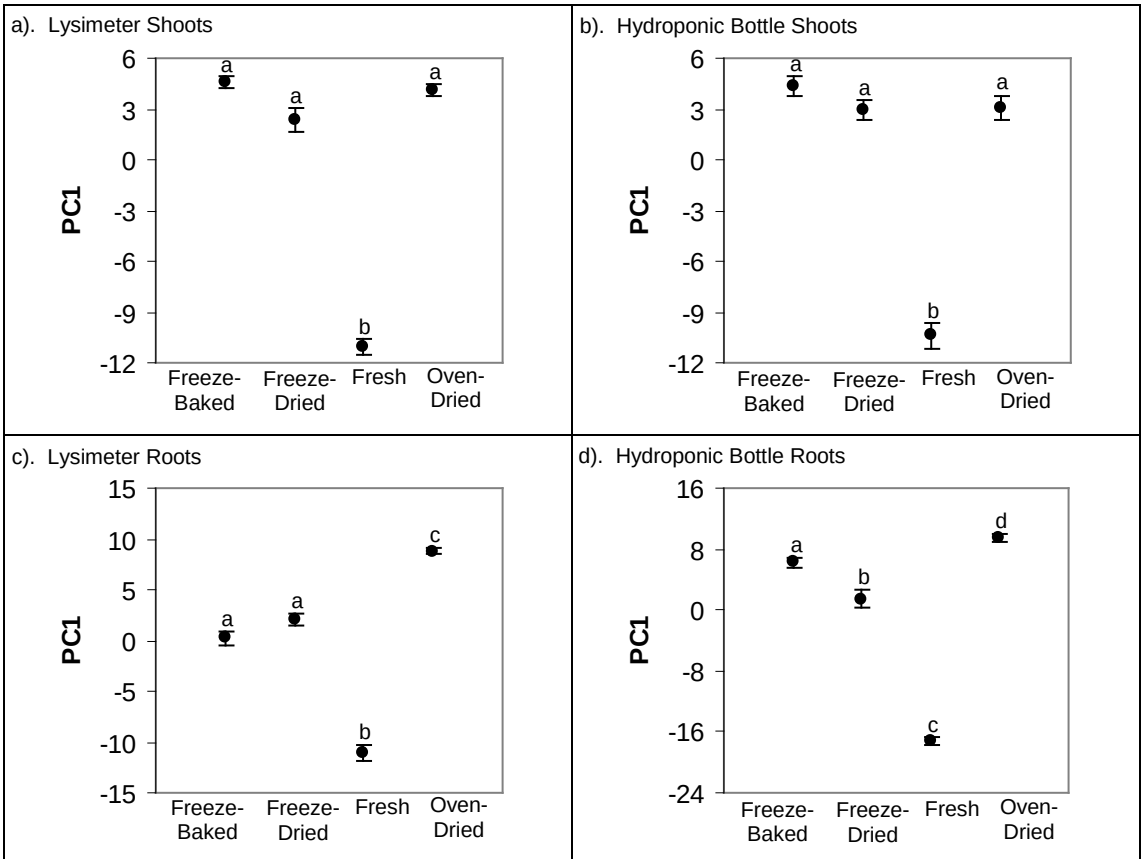


Figure 3.6. Effect of four storage types (freeze-baked, freeze-dried, fresh and oven-dried) on the metabolome of roots and shoots from plants grown in either lysimeters or hydroponic bottles (a-d). Different superscripted letters denote significant statistical differences of at least $p<0.05$. This format will be used throughout the thesis henceforth.

3.3.2.3: Flower Metabolome

The metabolome of the flowers was affected by UV-B which mirrors the pattern seen for the increased flowering rate under UV-B. Only fresh storage differed with oven-dried and freeze-dried being similar. Freeze-baking was not carried out due to the limited number of samples.

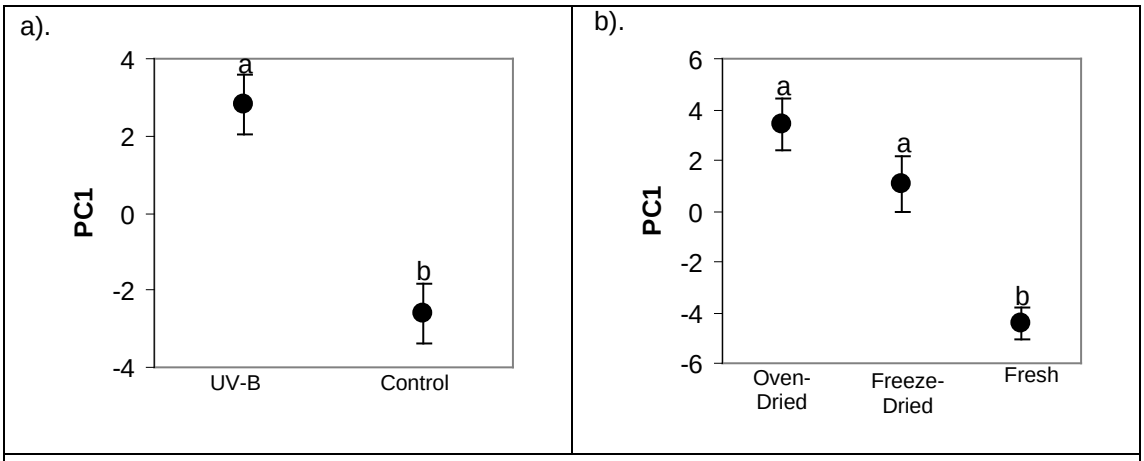


Figure 3.7. Effects of (a.) radiation and (b.) three storage types (irrespective of radiation) on flower metabolome.

3.3.2.4: Root Exudates

There was no effect of harvest or UV-B on the root exudates.

3.4: Discussion

3.4.1: *Initial Running of Blank Plates*

The results showed that running a blank plate prior to sampling and then accounting for this variation in the final analysis was not significantly different from running the plate without having run a blank plate initially (Figure 3.2). This suggests that it is possible to load the samples on to the plate without running the plate first as the reference well is sufficiently representative of all the others. This suggests the time taken to run an analysis can be halved and opens up the possibility of plating samples in a different location prior to analysis which would be of particular benefit to field-workers.

3.4.2: *Data Analysis*

The thesis will also analyse data using MANOVA or ANOVA should the first PC account for more than 95% of the variance. Whilst PC-DFA clustering techniques are the most widely used in metabolic fingerprinting (Fiehn, 2001), the use of MANOVA has been used in chemometric studies (Brereton, 1987) and favoured by some researchers using FT-IR (Arvanitoyannis & Nikolaos, 2005; Tso *et al.*, 2005; Gallo *et al.*, 2005). MANOVA and ANOVA also have the advantage that a probability value is more rigorous than judging differences by eye.

3.4.3: *Sample Storage*

The results showed that different storage techniques affected the metabolome (Figure 3.6). There is a lack of research conducted into studying the best storage methods for FT-IR of plant samples and as such these results are difficult to analyse in the context of other studies. However, the fresh sample technique was the most variable (possibly due to the uneven texture of the mixture) and was therefore not recommended. The dried samples were also different and showed differences in the effect of UV-B in the flowers (Figure 3.7). This suggests that freeze-drying does not necessarily have an advantage over oven-drying. A study looking at the best way to extract samples of DNA by Doyle & Dickson (1987) concluded that oven-dried samples often had the best preserved DNA and that this technique was superior to samples stored from chemically preserved samples. This also has further advantages to field-based researchers who could dry the samples on site for analysis at a later date. One advantage of freeze-drying was that the sample ground to a finer powder than the oven-dried samples which makes the sample easier to pipette and reduces the possibilities of an uneven surface affecting the results.

3.4.4: *The Effects of UV-B*

The low levels of UV-B used in this experiment (1.88 $KJ/m^2/hr^{-1}$) did not alter biomass in both syringe lysimeters and hydroponic bottles (Table 3.1). Whilst there is plenty of evidence to suggest that enhanced levels of UV-B can be damaging (Ballaré *et al.*, 1999) there are many sources which suggest plants are tolerant of UV-B and that the effects are likely to be small (Björn *et al.*, 1997; Allen *et al.*, 1999; Xu & Qiu,

2007). The second notable effect was that UV-B increased flowering in *L. japonicus* (Table 3.1) and altered the floral metabolome (Figure 3.7). Alterations to flowering and berry production have been observed in the sub-arctic dwarf shrub *Vaccinium myrtillus* (Gwynn Jones *et al.*, 1999b) due to enhanced UV-B although there have been studies that have shown UV-B to deleteriously affect flowering (Sampson & Cane, 1999). The alterations to the floral metabolome could be due to the fact that chalcone synthase production, which affects floral pigmentation, is altered by UV-B (Kreuzaler *et al.*, 1983) or that insects had indirectly contaminated the flowers having been attracted to the flowers reflecting UV-B light (Feldheim & Connor, 1996).

3.4.5: *Summary*

1. Running a blank plate prior to plating the samples had no advantage over running the plate with samples. In other words, the blank reference well is sufficiently representative of all the other wells to account for variation
2. Both cluster analysis and MANOVA/ANOVA analysis are fundamentally the same although MANOVA and ANOVA have the advantage that a probability value is given that there is no difference between the different treatments
3. Storage was shown to have an effect on the metabolome with fresh samples showing the most differences to the other samples which involved drying. Most importantly, UV-B differences were detected in the flower regardless of storage

Chapter Four: Development of a methodology to assess interference between *Lolium perenne* and *Lotus corniculatus*

4.1: Introduction

This chapter is concerned with the interaction between an artificial grass-legume system consisting of *Lolium perenne* and *Lotus corniculatus*. Grass-legume interactions are widely researched in plant interference experiments (Torssell, 1973; Lodge, 2002) given that such mixtures frequently exhibit facilitation (also called overyielding) whereby mixtures are more productive than the two respective monocultures (Springer *et al.*, 2001). This phenomenon has frequently been used to improve agricultural practice (Niang *et al.*, 1998) and has been attributed to the nitrogen-fixing capacity of the legume (Mendham *et al.*, 2004). However, in order to study this interaction it is necessary to use a suitable experimental design. This has been made difficult by the variety of different designs and the numerous advantages of each design.

The most common design has been the replacement series pioneered by De Wit in the 1960s. However, since the 1980s the validity of replacement series methodology has been questioned and numerous methodologies have radiated as a response. Gibson *et al.* (1999) concluded that it is unlikely a general consensus on which design to use will arise and that the design should depend on the hypothesis tested. Nonetheless, Gibson *et al.* (1999) strongly promoted the use of response surface methods, which will here be argued to be the most suitable for a general or preliminary study into inter-specific interactions.

It is initially worth justifying the use of greenhouse experiments as it has often been argued that such experiments are unrealistic and no inferences into natural processes can be gained (Gibson *et al.*, 1999). Whilst it is true that glasshouses lack realism they also have many benefits. For instance, the design can be more suitably planned for rigorous statistical analysis and any artificial environmental factors, for example UV-B levels, can be precisely administered. Furthermore, if interactions cannot be observed under glasshouse conditions they are unlikely to be of importance in natural communities (Gibson *et al.*, 1999).

Despite the unsuitability of de Wit replacement series (Inouyé & Schaffer, 1981; Joliffe *et al.*, 1984), they have still been extensively used by ecologists. Although there are numerous weaknesses in de Wit's methodology, it should be stated that all alternative models are variations on de Wit's basic theme. Connolly (1986) highlighted that size bias is an important flaw in replacement series because the analysis is biased towards the species with the largest biomass. Snaydon (1991) mentioned the futility of comparing oak trees with daisies as an example of this flaw. Replacement series also assume exact equivalence at the start of the experiment which is unlikely if the plants, which they naturally tend to be on germination, are of different sizes.

Another fundamental deficiency in replacement series is that the density of the plants is fixed. This neglects the fact that the plants may behave differently at varying densities. Firbank and Watkinson (1985) suggested avoiding this problem by growing two species in a 1:1 ratio at five densities. Similar experiments have been conducted

by Taylor & Aarssen (1989) and Austin *et al.* (1988); with the latter adding five different nutrient concentrations also. Such designs are usually referred to as additive designs as they are effectively replacement series at a variety of densities. Goldberg & Werner (1983) pioneered a similar approach which also incorporated focal species (an individual in middle of pot which is used to measure response) and associated species (those surrounding the focal species) as a basis for the model although Gibson *et al.* (1999) suggested that problems could arise if focal and associated species are varied simultaneously.

Another embellishment upon the additive model is the response surface model (Gibson *et al.*, 1999). This includes a range of densities, similar to the additive model, although it also incorporates a range of relative frequencies. Response models can be generated using general linear model analysis to fit regression models. Menchaca and Connolly (1990) used such a model to good effect showing that varying densities and frequencies results in a variety of responses. Law and Watkinson (1987) also showed the validity this technique by using *Phleum arenarium* and *Vulpia fasciculata*. Whilst such designs are more extensive they are the most useful in assessing the varying effects of densities and frequencies. Furthermore, they are more amenable to rigorous statistical analysis.

Temporal effects have also been neglected by competition studies. Goldberg and Barton (1992) noted that 63% of 89 field experiments reviewed by the authors only accounted for the effect at just one point in time and space only. If temporal effects are neglected then the changing dynamics of the interaction can easily be overlooked. Menchaca and Connolly (1990), in the aforementioned response model experiment,

also included week of harvest as a factor and noted a shift in the competitive balance between *Lolium perenne* and *Trifolium repens*. Turkington and Joliffe (1996) conducted a study that looked at botanically comparable pastures sown at around 1979, 1970, 1945 and 1880. The competitive balance of the similar species was shown to have changed over time showing that temporal effects have long-term consequences. Thus the effect of time must be included in all experiments if they are to be of any ecological benefit.

A related issue, raised by Gibson *et al.* (1999), is that there is a considerable difference between the outcome of competition and the effect of species on each other; a subtlety often overlooked by authors. If the final harvest is taken before the ultimate effect of competition is observed then the experiment cannot state with certainty what the ultimate nature of the interaction would be like although it will still offer important insights into the changing dynamics. Silvertown and Lovett Doust (1993) stated that the technical definition of competition is the effect on reproductive output although this is seldom measured. Moreover, the aforementioned experiment of Turkington and Joliffe (1996) shows that the effects could alter over a period measured in decades. Nonetheless, the distinction must be made.

In conclusion, the surface response model could be the most suitable design for an investigation looking to assess whether there is any competitive effect. Its incorporation of different densities and relative frequencies accounts for many of the deficiencies in replacement series methodology. The effects of time must also be incorporated as well as whether the effects are the ultimate effect of competition or a

transient effect of species on each other. Continued development of such designs can therefore give insights into any mechanisms proposed.

The following study presents two experiments that are based on the response-surface design of Connolly *et al.* (1990) using *Lolium perenne* and *Lotus corniculatus* (henceforth termed *Lolium* and *Lotus* respectively for simplicity). The first study was based in a glasshouse and the second was a continuation at a field-site to investigate how an outdoor environment may alter the interaction.

4.1.1: *Aims*

1. Test the hypothesis that mixtures of *Lolium perenne* (a grass) and *Lotus corniculatus* (a legume) will overyield (show facilitation) when grown in a mixture
2. Assess the suitability of response surface designs in ascertaining the competitive response and effect of both species in a mixture
3. Determine whether there is a difference in the interaction between the two species under glasshouse (experiment one) and outdoor (experiment two) conditions

4.2: Materials & Methods

4.2.1: *Response Surface Analysis*

The basic concept of a response-surface design is to create a regression model using different types of plant interference as components. For example, in most cases the variable that is to be modelled is the yield of an individual plant in a mixture. It is quite common to use inverse yield (reciprocal of biomass) as this reduces variation with an increase in weight (Menchaca & Connolly, 1990) although this was not carried out in this experiment in order to reduce artefacts from data manipulation. In a mixture containing two species, it can be deduced that two factors are affecting the yield of one individual in the mixture: (1) The density of other individuals of the same species and (2) the density of individuals of the other species. A model based on this would have the form:

$$Y_X = C_X + D_X A_X + D_Y A_Y \qquad (1)$$

In this equation, Y_X is the dependent variable of interest, namely the individual yield (Y) of species X in grams (hence Y_X). C_X is the constant and reflects the biomass of an individual where no competition is present; in other words the plant would weigh this much (g) if it was grown on its own in the same environment. The term $D_X A_X$ is the product of the density (D) of species X (hence D_X) and its coefficient (denoted A) of species X (hence A_X and $D_X A_X$ for the whole term). The density is simply calculated as the number of plants in the pot and the coefficient is effectively the amount of biomass lost for each individual of that species. For example, if the

coefficient was -0.1 and the density was 5 then the individual of interest would lose 0.5 g (from the constant) due to competition. The other species would also have a similar effect and this is reflected in the term $D_Y A_Y$ where D_Y is the density of the other species and A_Y is the coefficient. For example, if A_Y was -0.5 and there were 10 individuals of the other species ($D_Y = 10$) then the individual would lose 5 g.

There are therefore three terms of interest in the model: the constant, the density of the same species (henceforth the intra-specific coefficient) and the density of the other species (henceforth the inter-specific coefficient). Each of the terms is analysed and given a statistical significance with the significant terms being included in the mixture. Consider a model with a constant of 10 ($P<0.001$), intra-specific coefficient -0.5 ($P<0.001$) and inter-specific coefficient -0.01 ($P=0.998$). The constant and intra-specific coefficients are significant but the inter-specific coefficient is not and can be ignored. Therefore, if the individual was grown in a mixture with 10 similar individuals and 5 of the other species, there would be a loss of 5 g (10 x -0.5) as the five individuals of the other species effectively have no effect. In all experiments, polynomial equations were also used as the basis for the model although were never significant.

Data are presented in tables containing the three terms of the model for each species (constant, intra-specific, inter-specific) with the value of the term and significance value. The T-value is also presented to indicate data variability. Therefore, both the competitive response (the variable modelled) and competitive effect (the inter-specific coefficient) can be determined. This is advantageous as many designs can only determine competitive response without competitive effect (Goldberg & Landa, 1991).

When relevant, the coefficients are given for other factors such as harvest number and type of stress (e.g. enhanced UV-B and control).

The coefficients of response surface analysis are also of use as they form the basis from which to calculate species equivalence in the form of substitution rates. The substitution rate for a species X can be calculated as below (equation 2).

$$S_X = \frac{A_X}{A_Y} \tag{2}$$

The substitution rate (S_X) is effectively the quotient of the coefficients of the same (A_X) and different (A_Y) species. For example, if the intra-specific coefficient is -2 and the inter-specific coefficient is -1 then the substitution rate would equal 2. In other words this is how many individuals of the different species are required to have the same effect as one of the same species. It is clear from this simple example that the competing species has half the effect of the same species and thus double the number would give the same equivalence.

Throughout this project a systematic approach to choosing plant community structure was used which incorporated fixed densities (numbers of plants per pot) with different relative frequencies (proportion of pants in the pot). For example, three densities could be used such as 12, 8 or 4 plants per pot (as was typical in most experiments in this thesis). In most cases there were then five relative frequencies (ratio of species X and species Y: 100%:0%; 75%:25%; 50%:50%; 25%:75%; 0%:100%). Therefore, for three densities and five relative frequencies there would be fifteen communities with the precise number of individuals administered. Each materials and methods section

will (1) state the number and nature of the different densities used and (2) the number and nature of the relative frequencies.

4.2.2: *Experiment One: Glasshouse Design*

4.2.2.1: Community Structure

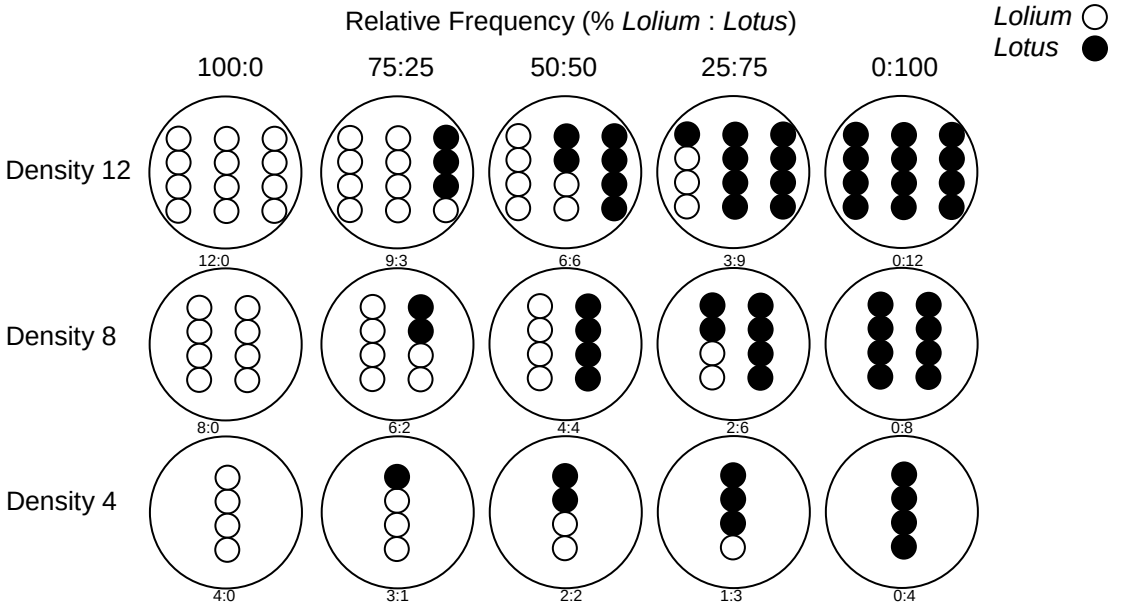


Figure 4.1. Schematic diagram showing the fifteen communities used and the number of *Lolium* and *Lotus* in each community (numbers shown under community). Seeds were sown in same positions although intermingled on germination and growth so that canopy was mixed (same for all subsequent experiments).

The first experiment was conducted over a seven-week period at the central glasshouse of The Botany Garden, Aberystwyth, between 4 October and 22 November 2004 under supplemental sodium lamps to extend lighting period for 12 hours. Fifteen artificial plant communities were chosen (Figure 4.1) containing *Lolium perenne* (grass) and *Lotus japonicus* (legume). Three densities were used (high - 12, medium - 8 and low - 4 plants per pot) with five relative frequencies (ratio

of *Lolium* and *Lotus*: 100%:0%; 75%:25%; 50%:50%; 25%:75%; 0%:100%). This allowed response surface analysis to be conducted according to previously described technique. The communities were replicated four times with five weekly harvests (15 x 5 x 4 = 300 experimental units).

Seeds of both species were sown in separate black plastic trays (30cm x 20cm x 5cm) filled with John Innes 1 seeding compost and covered by glass for germination. After one week the seedlings were pricked out and put into the relevant communities. Round 7.5 cm diameter pots (10 cm deep) were used with John Innes 3. In terms of density this relates to 14 cm^2 per plant in the high density (12 plants), 21 cm^2 per plant in the medium density (8 plants) and 42 cm^2 plants per pot at the low density (4 plants). The seeds were transplanted according to the design in Figure 4.1 although after two weeks the biomass had completely intertwined. After another week (two weeks after sowing), the seedlings were harvested for above and belowground biomass. This was repeated four more times at weekly intervals. Biomass was dried at 60º C and weighed for analysis.

4.2.2.2: Metabolic Fingerprinting of Plant Organs and Leachates

Plant material from both species was used from the dried harvest material for metabolic fingerprinting analysis. 0.5 g of both leaves and shoots (taken from the upper leaf blades or lowest parts of the roots) for all harvests were ground in a mill and mixed with 0.5 ml of distilled water for analysis by the IFS28 (see Chapter Two for methodology). For each species, samples were taken from monocultures and 50 % mixtures to see whether competition affects the metabolome. On the day before

the final harvest, the mesocosms were watered with 200 ml of distilled water and the leachates collected from drip-trays beneath the pots. The leachates for all 60 experimental units (15 communities x 4 replicates) were analysed by the IFS28 technique (Section 2.3.2.1).

4.2.3: *Experiment Two – Outdoor Design*

4.2.3.1: Community Structure

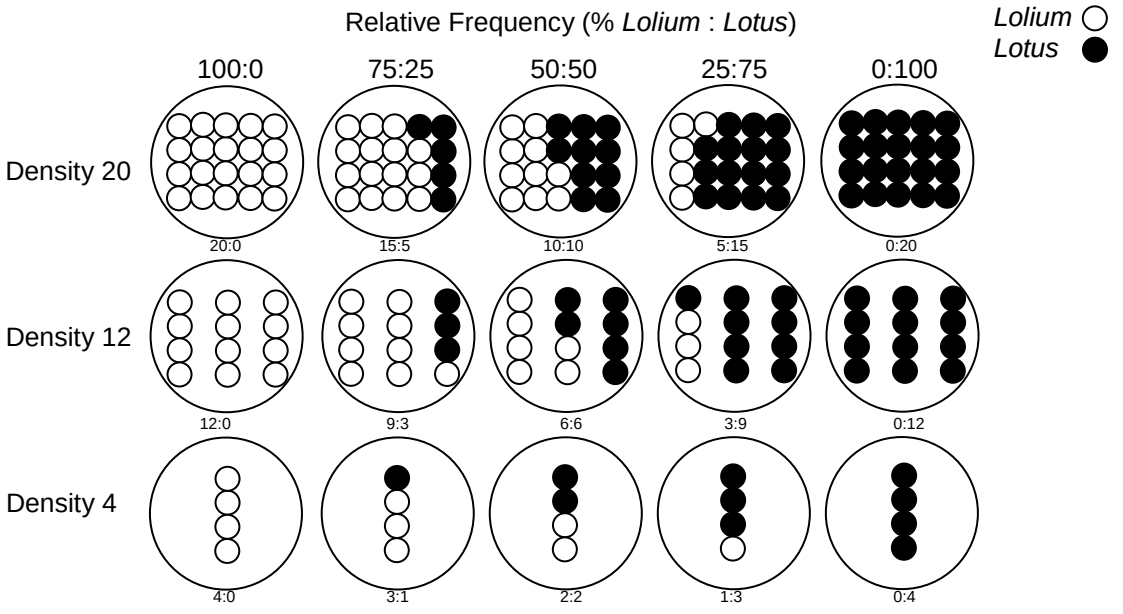


Figure 4.2. Schematic diagram showing the fifteen communities used and the number of *Lolium* and *Lotus* in each community (numbers shown under community).

A second experiment was conducted that aimed to use the same methodology as tested by the first experiment although in an outdoor setting. This was conducted over a 24 week period between 4 April 2005 and 19 September 2005 at the Trophic Interaction facility Field Site, Penglais Farm, Aberystwyth. Fifteen artificial plant communities were used (Figure 4.2) containing *Lolium perenne* and *Lotus*

corniculatus (legume). Three densities were chosen (high - 20, medium - 12 and low - 4 plants per pot) with five relative frequencies (ratio of *Lolium* and *Lotus*: 100%:0%; 75%:25%; 50%:50%; 25%:75%; 0%:100%). As a precursor to a proposed experiment involving 24 experimental blocks (conducted in next chapter) it was decided to use a replicate number of 24 (therefore 360 communities in total) which would substantially reduce standard error.

Another key difference between the experiments was the harvest structure. Instead of harvesting the whole plant (leaves and roots) only the upper leaves were harvested (2 cm above ground level). The plant was then left to regrow and this subsequent (regenerative) growth was harvested. This is typical of such experiments (Menchaca & Connolly, 1990) and was chosen as it would be almost impossible to differentiate between the two root systems of the species after an extended period of time. Despite *Lotus* spp. being dicotyledonous, the plant was able to regenerate from nodes near the base of the plant and was therefore not detrimentally affected by the harvest. Three harvests were conducted at eight-weekly intervals from the start of the experiment.

The seeds were directly sown in excess into 15 cm diameter black pots filled with John Innes 3 compost at the central glasshouse of The Botany Garden, Aberystwyth. The pots were covered with black plastic for one week for germination. The seedlings were then thinned out until they represented the communities illustrated in Figure 4.2. As with the glasshouse experiment, they were sown according to the design on Figure 4.2 although after two weeks the seedlings had completely intertwined. In terms of density per area in the pot, the high density (20 plants) represented one plant per 9 cm^2, the medium density (12 plants per pot) represented

one plant per15 cm^2 and the low density (4 plants per pot) represented one plant per 44 cm^2.

4.2.3.2: Metabolic Fingerprinting of Plant Organs and Leachates

Dried leaf material (0.5 g) was mixed with 0.5 ml of distilled water for analysis using the IFS28 technique. Separate analyses were carried out for the three harvests and replicated four times. Leachates were sampled from the drip trays beneath the pots on the day of the harvest. 500 ml of water was poured on the pots and the leachates sampled after one hour. The plants were lightly watered the day before to ensure that they had not dried out as any cracks in the soil would allow the water to pass straight through to the drip tray. Care was taken to ensure watering was not too heavy as this may wash away any root exudates. Leachates were analysed by the IFS28 technique.

4.3: Results

In both experiments the *Lotus* plants showed nodulation. The nodules were easily observed on the surface of the soil and if some soil was pushed aside it could be found that nearly all the roots had bunches of nodules on them. A drawback of the design was that no assay was taken to confirm this although the extensive nodulation and rapid growth of the plants suggest that nodulation was indeed occurring.

4.3.1: *Experiment One – Glasshouse Design*

4.3.1.1: Community Biomass

There was a clear effect of harvest time on community productivity with a predictable increase in biomass over time ($P<0.001$). There was no difference in aboveground biomass between the final two harvests and it appears a maximum community biomass had been reached (Figure 4.3a). This pattern was not observed belowground with biomass increasing over the duration of the experiment (Figure 4.3b).

The three densities had no effect on both aboveground and belowground community biomass ($P<0.001$) (Figure 4.3). Therefore, a community with 4 plants was just as productive as a community with 12 plants. From this fact alone it can be deduced that competition lowered the biomass of the individual species.

However, the community structure did have a significant effect on the overall biomass. Apart from the first harvest where there were no differences ($P=0.679$), the *Lotus* monoculture had the lowest biomass with all other communities having a similar biomass.

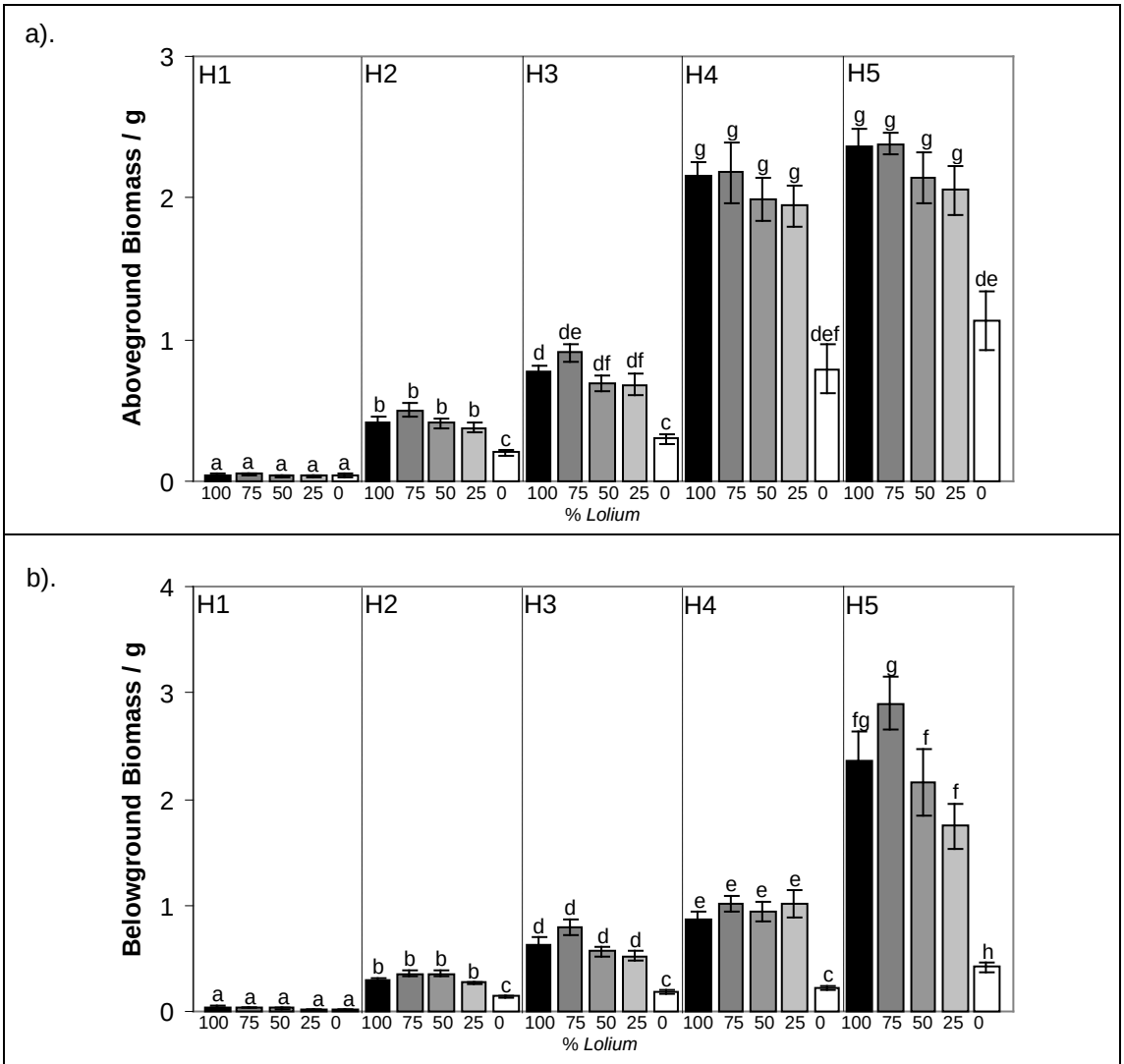


Figure 4.3. Effect of species composition on total community biomass above- (a) and belowground (b). The five columns represent the five harvests with number after H denoting week after communities formed (n=4).

4.3.1.2: Plant Interference

Analysis of the constants (Table 4.1) shows that there is an increase in individual biomass over time (Figure 4.3). The competition coefficients did not vary which suggests that the intensity of competition was uniform regardless of size of the plants. This pattern was mirrored for both belowground and aboveground parameters (Table 4.1).

Table 4.1. Table showing the competition coefficients for *Lolium* and *Lotus*. Coefficients are presented for below- and aboveground organs for all five harvests (weeks after communities were formed). No substitution rate is given as the inter-specific coefficient is never significant. The T-value is shown in parentheses with significance denoted by superscripted asterisks.

Aboveground				
Species	Harvest	Constant	Intra-specific	Inter-specific
Lolium	1	0.01 (10.3)***	0.00 (0.1)^{ns}	-0.00 (-2.0)^{ns}
	2	0.10 (13.3)***	-0.01 (-5.9)***	-0.00 (-0.5)^{ns}
	3	0.23 (11.7)***	-0.02 (-6.0)***	-0.00 (-0.2)^{ns}
	4	1.00 (10.6)***	-0.09 (-6.9)***	-0.02 (-0.1)^{ns}
	5	0.88 (12.8)***	-0.07 (-7.7)***	-0.00 (-0.7)^{ns}
Lotus	1	0.00 (1.9)^{ns}	0.00 (0.7)^{ns}	0.00 (1.2)^{ns}
	2	0.08 (10.2)***	-0.01 (-6.2)***	-0.00 (-0.1)^{ns}
	3	0.11 (10.0)***	-0.01 (-5.5)***	-0.00 (-1.4)^{ns}
	4	0.13 (5.6)***	-0.01 (-1.5)^{ns}	-0.00 (-0.4)^{ns}
	5	0.20 (8.3)***	-0.01 (-3.0)**	-0.01 (-1.7)^{ns}
Belowground				
Lolium	1	0.00 (5.9)***	0.00 (1.8)^{ns}	0.00 (0.5)^{ns}
	2	0.09 (12.3)***	-0.01 (-6.4)***	-0.00 (-0.5)^{ns}
	3	0.18 (9.5)***	-0.01 (-4.5)***	0.00 (0.1)^{ns}
	4	0.42 (9.7)***	-0.04 (-6.5)***	-0.00 (-0.3)^{ns}
	5	0.93 (8.7)***	-0.07 (-4.5)***	-0.01 (-0.7)^{ns}
Lotus	1	0.00 (4.1)***	0.00 (0.4)ns	-0.00 (-0.9)^{ns}
	2	0.07 (10.9)***	-0.01 (-7.5)***	-0.00 (-0.8)^{ns}
	3	0.08 (11.3)***	-0.01 (-6.7)***	-0.00 (-0.7)^{ns}
	4	0.11 (4.8)***	-0.01 (-2.9)***	-0.00 (-0.6)^{ns}
	5	0.15 (9.9)***	-0.01 (-6.3)***	-0.00 (-1.1)^{ns}

All inter-specific coefficients were not significant suggesting that *Lolium* has no effect on *Lotus* and vice versa (Table 4.1). Both act as if the other species was not

present. All intra-specific coefficients were significant except for the first harvest and aboveground biomass of *Lotus* in the fourth harvest. This suggests that competition within species is far more intense than between species.

4.3.1.3: Metabolic Fingerprinting of Above- and Belowground Organs

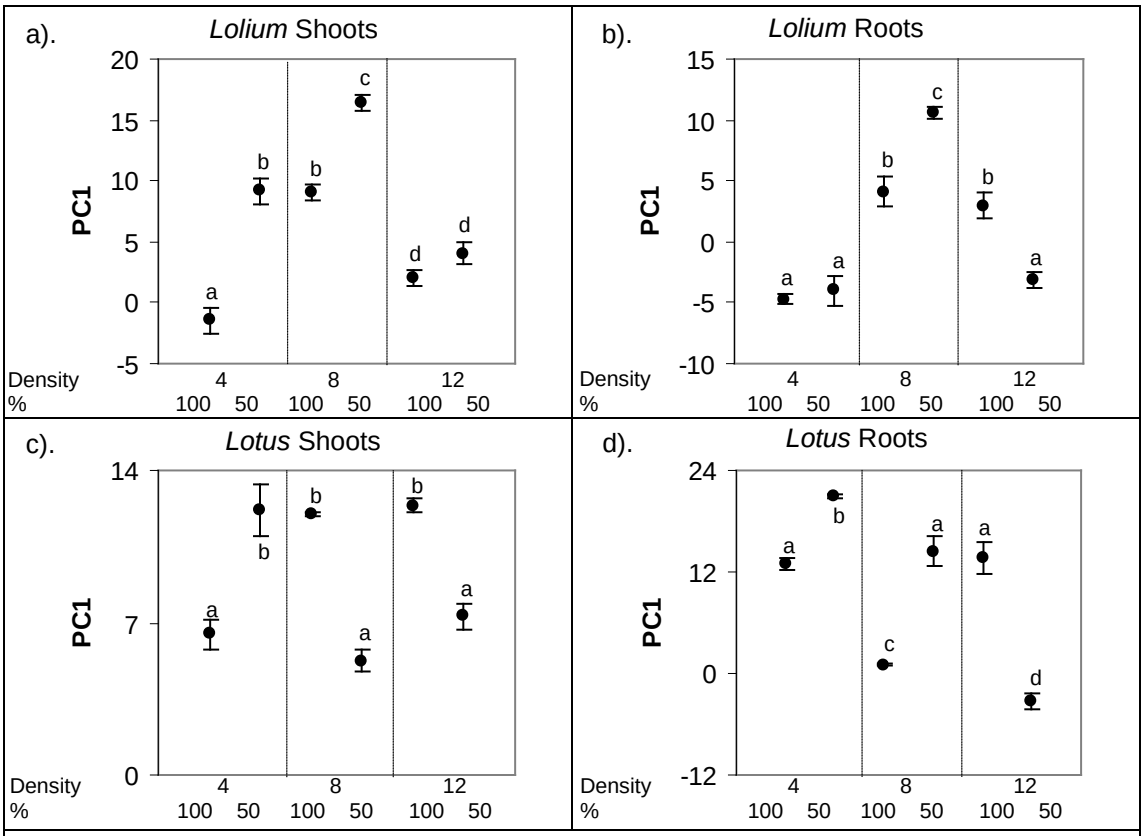


Figure 4.4. Effect of density and competition on the metabolome of shoots and roots (columns) in *Lolium* and *Lotus* (rows). Letters above standard error bars denote statistical similarities and differences.

Whilst density did not have an effect on the biomass of communities (Figure 4.3), it did have a significant effect on the metabolome of the different plant species. Moreover, in 10 of the 12 cases, the metabolome from a plant in a monoculture (frequency of 100 %) was different from a plant in a binary mixture (frequency of 50 %). This suggests that competition has an effect on the metabolome.

However, as can clearly be seen from Figure 4.4, this phenomenon was only observable in isolation for a specific organ and density. For example, there was a clear difference between *Lolium* shoots in a mixture and in monoculture at a density of four plants per pot. However, the profile of the plant in a mixture was exactly the same as the profile for the plant in monoculture at a density of eight plants per pot. Therefore, whilst competition did affect the metabolome, it was clear that the chemical changes were not sufficiently uniform on which to base predictions about the type of competition.

4.3.1.4: Metabolic Fingerprinting of Leachates

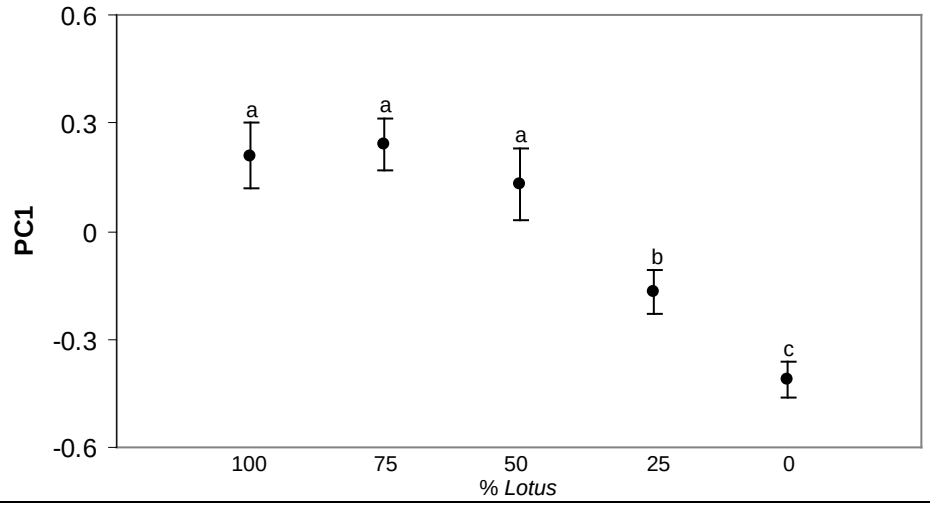


Figure 4.5. Alteration in metabolic fingerprinting with change in *Lotus* abundance

The metabolic profile of the leachates was unaffected by density although it was by the community composition (Figure 4.5). This mirrors the pattern observed for aboveground community biomass (Figure 4.3). Furthermore, it can be seen from Figure 3.3 that there was an alteration in metabolic profile with community structure.

4.3.2: *Experiment Two – Outdoor Design*

4.3.2.1: Community Biomass

As with the glasshouse experiment, the density of plants per pot had no bearing on the community biomass. The second harvest (thus first regenerative growth) was the most productive although the second and third (thus second phase of regenerative growth) harvests had a similar biomass (Figure 4.6).

A facilitative effect can be observed on Figure 4.6 for all three harvests with the mixtures yielding more than the two monocultures.

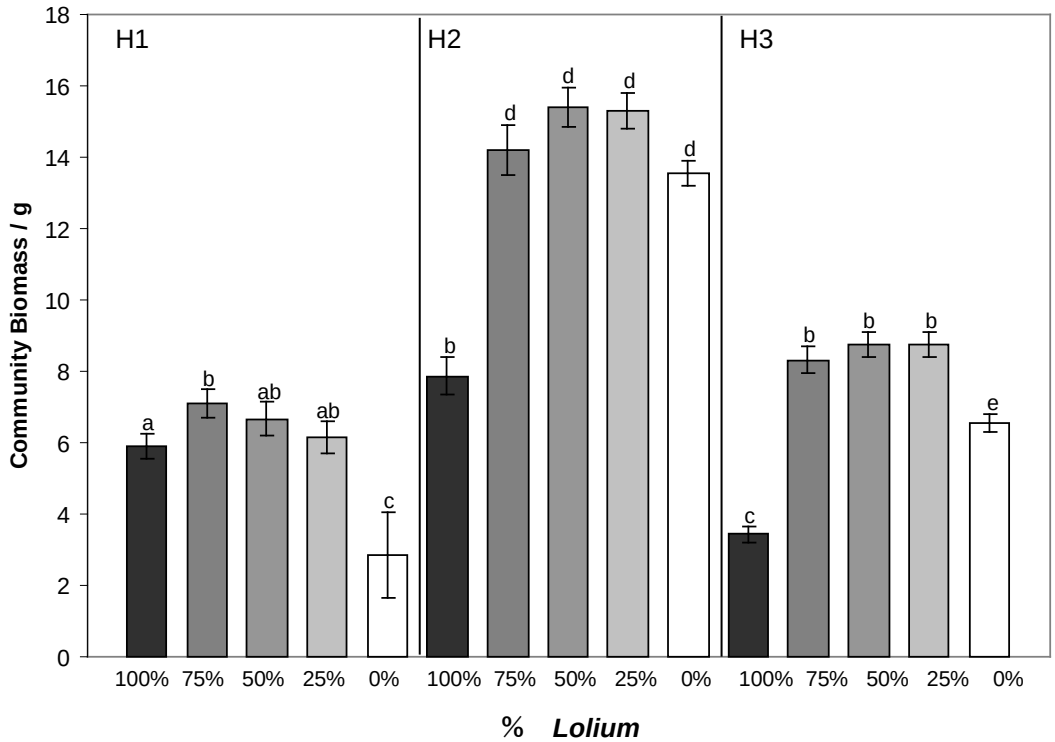


Figure 4.6. Total community biomasses for the three harvests (H1 to H3). All densities were combined to show *Lolium* % (the remaining % being *Lotus*). The mixtures (in grey) are always greater than the monocultures (solid bars).

4.3.2.2: Plant Interference

Table 4.2 presents the competition coefficients for the two species and all harvests. The greatest constant for *Lolium* was in harvest two which was to be expected given this was the most productive harvest although no such pattern was observed for *Lotus*.

All intra-specific and inter-specific coefficients were significant suggesting each species was affected not only by the same species (as in the glasshouse experiment) but also by the other species. The substitution rates for both species were similar (range 0.3 to 0.6) suggesting that each species had a similar effect on each other. The fact that all substitution rates were lower than one suggests intra-specific competition is more intense than inter-specific competition. This can clearly be seen in Table 4.2 as the intra-specific coefficients were greater than the inter-specific coefficients. Whilst the coefficients and substitution rates vary slightly with time, the effects do not obscure the fact that both species have a similar equivalence.

Table 4.2. Table showing the competition coefficients for *Lolium* and *Lotus* over the three harvests. The T-value is shown in parentheses with significance denoted by superscripted asterisks. The substitution rate in final column indicates species equivalence. In all cases the intra-specific coefficient is greater than the inter-specific.

Species	Harvest	Constant	Intra-specific	Inter-specific	Sub Rate
Lolium	1	1.98 (25.7)***	-0.10 (-15.0)***	-0.05 (-5.6)***	0.46
	2	6.72 (27.9)***	-0.39 (-18.4)***	-0.15 (-5.7)***	0.38
	3	2.07 (22.0)***	-0.11 (-13.3)***	-0.06 (-6.4)***	0.59
Lotus	1	1.23 (18.0)***	-0.07 (-11.3)***	-0.03 (-4.7)***	0.51
	2	2.18 (22.1)***	-0.11 (-13.2)***	-0.08 (-7.4)***	0.69
	3	2.81 (19.9)***	-0.17 (-13.8)*	-0.05 (-3.1)**	0.28

4.3.2.3: Metabolic Fingerprinting

Only the third harvest showed significant differences in the leaf metabolome for both species (Figure 4.7). In both cases, there were alterations in metabolome due to competition. For *Lolium*, the monocultures were clearly distinct from the low frequency stands with 50 % and 75 % *Lolium* frequencies being similar. The pattern was less evident for *Lotus*, although despite 50 % and 25 % frequencies being identical, there appeared to be a similar shift in metabolome with competition also. Therefore, it is clear in the second harvest (where biomass changes were greatest) that competition was chemically modifying the leaves although the extent is still unknown.

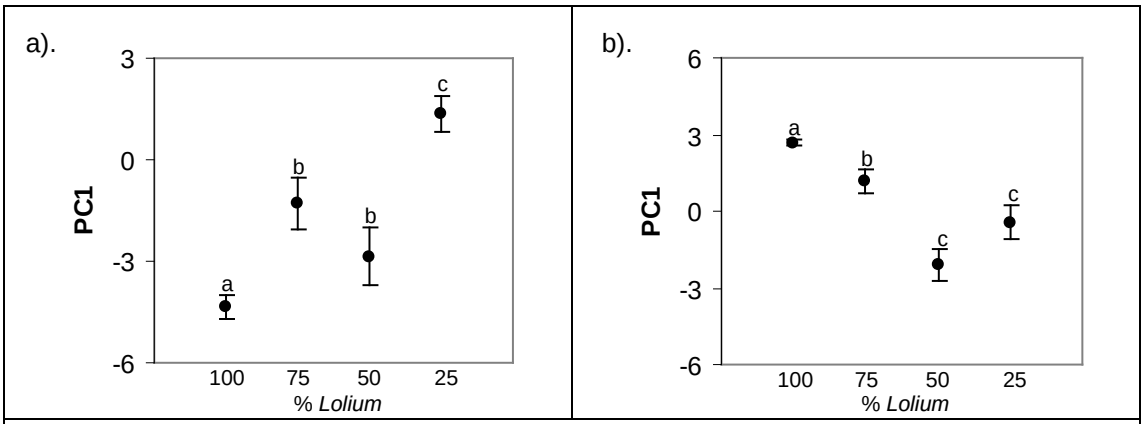


Figure 4.7. Effect of competition on the metabolome of *Lolium* (a.) and *Lotus* (b). in the second harvest. Both species' metabolomes are plotted against the frequency of *Lolium* (the remaining being for *Lotus*).

There were no significant differences in the chemical fingerprint of the soil leachates for harvest or community composition.

4.4: Discussion

4.4.1: *Suitability of Response Surface Designs*

The central advantages of using response surface analyses are that the densities of the two species can be varied independently and that the competition coefficients can explain the competitive effect and response in terms of biomass loss (Tables 4.1 and 4.2). Another advantage of response surface analysis is that the substitution rates help reduce size bias (Connolly & Wayne, 1996; Connolly *et al.*, 2001b). Size bias is a central problem in competition research (Grace *et al.*, 1992; Gibson *et al.*, 1999) as the largest plant is taken to be the 'winner' when biomass is used as the measure of competition (Freckleton & Watkinson, 2001). This was not a problem in these experiments as the biomasses for both *Lotus* and *Lolium* were similar (Figures 4.3 and 4.6). Substitution rates are effectively the ratio between the intra- and inter-specific effects and therefore should a larger species (such as a tree) exert a small effect on a smaller species (such as a grass) this would be reflected in the smaller substitution rate and not the large size of the competitor. Given abiotic stress may considerably alter the differences in biomass between plants then substitution rates are a suitable precaution against this form of bias.

Goldberg *et al.* (1995) mentioned that a key problem in stress competition is that competition is seldom directly measured in such experiments. By comparing competition coefficients from plants under stress (providing both sets of coefficients were significant) it is possible to see how competition is changing due to stress. To corroborate this, there have been studies using response surface analysis that have

successfully tested the suitability of this design (Wayne *et al.*, 1999; Nolan *et al.*, 2001). Response surface designs could therefore be of great use in assessing the effects of abiotic stress, especially with the pressing need to find a suitable experimental design in climate change experiments (Teughels *et al.*, 1995).

Time is also an important factor to consider in experimental design (Hutchings & Budd, 1981) although most studies only concentrate on one harvest during the early stages of the interaction (Goldberg & Landa, 1991). The glasshouse experiment was conducted over a short period of time (seven weeks) and still showed competitive effects. Table 4.1 shows how there was no competition in the first harvest although it was present thereafter. However, after the first harvest it was clear that the pattern (where intra-specific was greater than inter-specific competition) was established and this did not vary. The outdoor experiment showed no change in pattern over the course of the experiment although biomass did change (Figure 4.6; Table 4.2). Density did not affect the interaction either which is contrary to other studies (Connolly *et al.*, 1990; Turkington & Joliffe, 1996). It therefore appears that the main competitive effects are established after a brief period of competitive alteration. This suggests that having a limited number of harvests poses no problems. However, it should be noted that abiotic stress can affect competition depending on time (Menchaca & Connolly, 1990; Wayne *et al.*, 1999). Therefore, it was decided for future experiments that three harvests for an outdoor experiment was sufficient.

Another complication surrounding harvest collection is whether the harvest was completely destructive (above- and belowground) or whether only the aboveground biomass is harvested to allow for regenerative growth. The first experiment opted for

the former option and gained a more complete understanding of the interaction by obtaining root biomass. However, the effects of the roots mirrored aboveground processes and as such did not bring any extra information to the study. Furthermore, by using regenerative growth it was possible to maximise available space without having to account for harvests that will be removed. A study by Menchaca and Connolly (1990) also showed that regenerative growth harvests were tenable using the response surface approach. However, it has been shown that clipping can cause differences in interclonal competition using response surface analysis (Bullock *et al.*, 1994). Nonetheless, it appears that little information is lost when regenerative growth is used and can thus be used in future experiments.

4.4.2: *The Facilitative Effect*

Whilst measuring total community biomass does not require any statistical modelling, it is still highly important as it shows which combinations of plants are the most productive (Rajaniemi & Goldberg, 2000; Gough *et al.*, 2001). Figure 4.6 shows a clear facilitative effect with mixtures yielding more than monocultures. Under glasshouse conditions (Figure 4.3) it is clear that mixtures are more productive than *Lotus* monocultures but not pure stands of *Lolium*. In both cases, it is clear that the initial species composition was instrumental in ascertaining the final community biomass. The fact that the initial seeding ultimately determines community biomass does not preclude the presence of competition which clearly affects individual biomass (Tables 4.1 & 4.2). This phenomenon, where strong competition on individuals occurs without impacting on community biomass has also been noted by

Connolly and Wayne (2005) and Ramseier *et al.* (2005). Therefore, calculating community biomass is essential in all competition experiments.

The density of a community is also important in determining the final biomass (Shilo-Volin *et al.*, 2005). However, density did not have an effect in these experiments; the pots supported a critical biomass that was not altered by the number of individuals in the pot. It has also been argued that density can affect temporal responses (Goldberg *et al.*, 2001) although this was also not observed from the result (Figures 4.3 and 4.6). Nonetheless density is still an important factor to use in further experiments in this thesis as it has been shown to affect susceptibility to stress (Fowler, 1995) and in either case a response surface analysis requires at least two fixed densities in order to be viable. Therefore, future experiments will use two densities (low and high) to allow detection of possible effects whilst removing unnecessary replication.

The clear facilitative effect observed from the community biomass from the outdoor experiment (Figure 4.6) is only to be expected given the fact that most grass-legume mixtures overyield (Luscher *et al.*, 1992; Connolly *et al.*, 2001a; Gosling, 2005). Analysis of the competition coefficients gives an indication of the mechanistic basis behind this phenomenon. Surprisingly, none of the coefficients were positive which is to be expected when one assumes that legumes are positively benefiting the grass. Instead it appears that both species are negatively affecting each other. However, this is still compatible with the occurrence of facilitation provided the intra-specific coefficients are greater than the inter-specific coefficients which was found in both studies (Tables 4.1 & 4.2). It is clearly beneficial for a plant to be grown amongst different species when intra-specific competition is more intense than intra-specific

competition as the contention for the same resources is more severe. Therefore, both species are not competing so intensely in mixtures compared to when they were grown in pure stands.

This is at odds with the widely held view that facilitation between grasses and legumes is due to the nitrogen-fixing capacity of legumes inadvertently benefiting other species (Dupraz *et al.*, 1998; Li *et al.*, 2003; Rodriguez-Echeverria *et al.*, 2003). Such a rationale was even the original hypothesis of this project as argued in the introduction. However, it has been shown that legumes can act as competitors (Marquez & Allen, 1996) and the central conclusion from a study by van Ruijven and Berendse (2003) was that legumes are not the only facilitators. Given that mixtures lower the more intense intra-specific competition, many ecosystem modellers have concluded that most species have the capacity to overyield (Hooper & Dukes, 2004; Lambers *et al.*, 2004; Beckage & Gross, 2006). Research using resource complementation analysis have shown that mixtures are more effective at maximising the available resources whilst monocultures are more selective and conclude that this is the basis of overyielding (Putnam & Allan, 1992; Ayisi *et al.*, 1997; Skelton & Barrett, 2005). Another approach has highlighted the role of different functional types in overyielding mixtures (Tilman *et al.*, 1997; Wright *et al.*, 2006). Different functional groups such as grasses, forbs, legumes and creeping non-legume forbs (Lanta & Leps, 2006) are more effective at utilising available nutrients thus allowing facilitation to occur. Whilst only two species were used in this study both were from different functional groups. It is therefore more than possible that facilitation between a grass and a legume is unrelated to nitrogen fixation as shown in this study.

However, the idea that facilitation is the direct cause of nitrogen fixation cannot be completely rejected as there is a large body of evidence to suggest nitrogen is increased in grasses grown amongst legumes (Carter & Ambus, 2006; Moyer-Henry *et al.*, 2006; Rasmussen *et al.*, 2007). Furthermore, it has been directly shown using N-15 labelling, that nitrogen from legumes is transferred to other species (Høgh-Jensen & Schjoerring, 2000; Høgh-Jensen, 2006). It is therefore possible that fixed nitrogen is shared between the two species in this experiment; especially as nitrogen sharing is common amongst all species (Fargione *et al.*, 2007). However, this does not necessarily mean that facilitation is a direct result; such evidence would in either case be correlative. Nevertheless, many reviews on this subject explicitly attribute facilitation to nitrogen sharing (Hauggaard-Nielsen & Jensen, 2005; Høgh-Jensen, 2006) and with no similar review from the alternative viewpoint highlighted by this project, the data remain controversial. It is hoped that more discussion and research will lead to a clearer understanding of the underlying mechanism.

Metabolomic fingerprinting was also of limited use and will be discussed in the context of all other studies at the end of the thesis. However, the main patterns were that leachates reflected community structure (Figure 4.5) in the glasshouse but not outdoors (Figure 4.1). Analysis of the leaves showed shifts in metabolome with altered competition although this was only for a limited number of cases (Figures 4.4 & 4.7). Thus there were no repeatable patterns from which to base conclusions at this stage.

4.4.3: *Conclusion*

In conclusion, these experiments have shown that response surface analysis is a suitable technique for basing competition experiment and will be apt for studies using abiotic stress. It is clear that *Lotus-Lolium* mixtures exhibit facilitation and the reasons for this appears to be more related to lowered intra-specific competition as opposed to the nitrogen-fixing ability of the legumes.

4.4.4: *Summary*

1. Response surface designs are effective in assessing the competitive response and effect of *Lolium perenne* and *Lotus corniculatus* and the technique can be used in future experiments
2. *Lolium perenne* and *Lotus corniculatus* show a facilitative effect and therefore present a suitable model system for testing the effects of UV-B on this phenomenon
3. The basis of overyielding appears to be due to a decrease in competitive pressure in mixtures as opposed to monocultures

Chapter Five: The effects of enhanced UV-B on the interaction between *Lolium perenne* and *Lotus corniculatus*: A test of the theories of Tilman and Grime

5.1: Introduction

This section studies the effects of UV-B on the model grass-legume system described in the previous chapters. Response-surface analysis is applied to see whether competition is always present (Tilman, 1985) or only under conditions of no stress (Grime, 1977). The results are thus analysed in the context of similar studies and contribute to the Tilman-Grime debate. Consideration is also given as to whether UV-B is affecting belowground leachates and the metabolomic techniques used to test this are discussed for their suitability.

5.1.1: Aims

1. Test whether enhanced UV-B alters the competitive interaction between *Lolium perenne* and *Lotus corniculatus*
2. Assess whether soil leachates are altered by enhanced UV-B using FT-IR
3. Test whether competition ceases under conditions of stress (thus testing theories of Tilman and Grime)

5.2: Materials & Methods

5.2.1: *Experimental Set-Up*

The experiment was conducted over a 24 week period between 3 April 2006 and 18 September 2006 at the Trophic Interaction Facility Field Site (set-up of site described in Chapter Two).

The experiment investigated the effects of three treatments (UV-B, UV-A and control) on *Lolium perenne* and *Lotus corniculatus* mesocosms. Community structure was designed to allow for regression modelling by response-surface analysis. Five relative frequencies (*Lolium*:*Lotus* - 100:0; 75:25; 50:50; 25:75; 0:100) and three densities (20, 12, 4) were used.

Soil media and seed providence was also the same as in the previous section. The seeds were sown in excess and thinned out one week later (germination being around three days). After two weeks the communities were moved outside to acclimatise. After one month the communities were taken to the field site for the start of the radiation treatment. The first full-aboveground harvest was taken eight weeks after the start of the experiment (therefore after four weeks radiation) with two further harvests taken after eight-week intervals. As with the outdoor experiment in Chapter Four, the same pots were used for the harvests and thus regenerative growth was harvested.

5.3: Results

As with the previous experiments, the *Lotus* plants showed extensive nodulation.

5.3.1: *Community Biomass*

Results from community biomass are presented in Figure 5.1. The results suggest that the interaction varied considerably over time. In the first harvest, the range of biomasses varied from 6 g to 12 g for all treatments whilst in the last two harvests, the biomasses ranged from less than 1 g to 4 g. This was because the biomass was harvested from the same pots as the first harvest (using regenerative growth) and explains the reason why the biomass had decreased. This shows that with each successive harvest, the biomass decreased considerably regardless of community structure or UV-B stress.

Moreover, in the first harvest there was a clear facilitative effect with the monocultures for both species being a similar weight. The monocultures were on average 100 % lower than the biomasses of the mixtures which were similar regardless of community structure. In the second harvest the UV-A and control showed an increase in biomass with an increase in *Lotus* biomass. UV-B had no effect. However, by the third harvest a more rigid pattern had emerged. For the UV-A and control treatments, it was only the *Lolium* monoculture that was significantly lower whilst under UV-B it was only *Lotus* monocultures that were detrimentally affected.

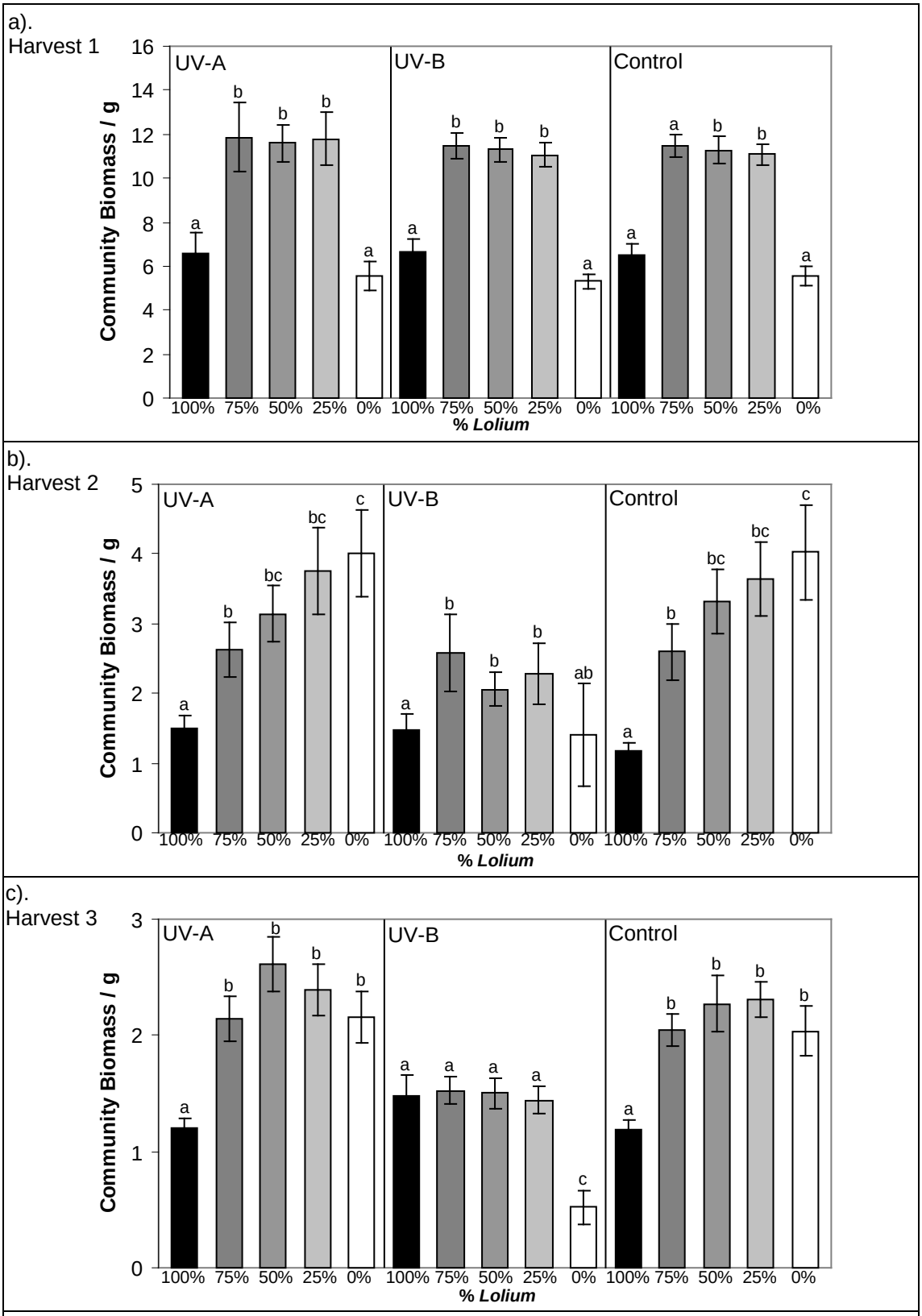


Figure 5.1. Effects of enhanced UV-A and UV-A radiation on community biomass over the three harvests. All densities were combined to show just % of *Lolium* (the remaining % being *Lotus*). Biomass of all communities decreased over each harvest (see horizontal axis). Different letters denote statistical differences ($p<0.05$).

The overall pattern from Figure 5.1 is that the community biomass varied over time from a facilitative situation, through a transitional period (harvest 2) until it was only the monocultures that were most affected by the environment conditions. In the case of *Lotus*, this was most notable under UV-B with a monoculture biomass of less than 1 g compared to the 6 g monocultures at the start of the experiment.

5.3.2: *Plant Interference*

Table 5.1 presents the competition coefficients and substitution rates for both species over the three harvests and for the three radiation treatments. Analysis of the constants in the third column is useful as they give an indication of the size of an individual plant for any given treatment regardless of competition. It is evident that the first harvest was more productive than the second and third harvests which were similar. The most productive species was *Lolium* with both species benefiting from UV-A radiation. However, in the last two harvests, UV-B appeared to benefit *Lolium* (a constant of 1.08 compared to 0.92 for UV-A and 0.8 for control) although was detrimental to *Lotus* (0.41 for UV-B compared to 1.44 for UV-A and 1.35 for control). The increase in *Lolium* biomass was not necessarily because the biomass of *Lotus* decreased. This was because the inter-specific coefficient for *Lolium* under UV-B was higher than for the other conditions. This suggests that UV-B had a differential effect on the two species in the later stages of competition.

Analysis of the intra-specific coefficients also provides evidence that UV-B was altering plant interactions. In the first harvests, however, UV-B did not affect intra-specific competition for either species. *Lolium* had the greatest coefficients (-0.21 on

average) compared to *Lotus* (-0.17 on average) despite similar constants. This suggests that *Lotus* was less affected by competition than *Lolium*. In the second and third harvests it appeared that *Lotus-Lotus* interactions were significantly affected by UV-B although *Lolium-Lolium* interactions were not. The intra-specific coefficients for *Lotus* decreased significantly (-0.02 compared to -0.07 for the controls) suggesting competition decreases under conditions of stress.

The inter-specific coefficients also showed a similar pattern to the intra-specific coefficients. In the first harvest, radiation appeared to have no effect on the interaction with the lowest coefficients for *Lotus* which suggests that *Lotus* is less affected by *Lolium* than *Lolium* is to *Lotus*. The final two harvests showed a similar pattern to the intra-specific coefficients, namely that *Lolium* remains unaffected by UV-B whilst *Lotus* is damaged. This suggests that under UV-B, *Lotus* is unaffected by both different and similar species. This lends weight to the theory that competition is unimportant under conditions of stress.

The substitution rates also support this view. In the first harvest there was little difference between species or treatment. All the rates were close to 0.5 which suggests that the competing species has half the effect the same species has. This clearly shows that intra-specific competition is far stronger than inter-specific competition. However, in accordance with the other coefficients, the second and third harvests show a departure from the first harvest. Substitution rates were higher for *Lotus* than *Lolium* which suggests a subtle shift in favour of grasses over time. Furthermore, it was not possible to calculate the substitution rates under UV-B for

Lotus as competition had ceased to be of importance. This again clearly shows that UV-B lessens the effects of competition.

Table 5.1. Table showing the competition coefficients for the two species divided according to harvest and radiation. The T-value is shown in parentheses with significance denoted by superscripted asterisks. In all cases (except the control *Lotus* in the final harvest) the intraspecific coefficient is greater than the intraspecific coefficient. The substitution rate in the final column indicates species equivalence.

Harvest One					
Species	Radiation	Constant	Intraspecific	Interspecific	Sub Rate
Lolium	UV-A	4.32 (13.0)***	-0.23 (-8.3)***	-0.11 (-3.2)**	0.48
	UV-B	3.95 (14.9)***	-0.21 (-9.0)***	-0.10 (-3.5)***	0.48
	Control	3.86 (15.0)***	-0.20 (-9.1)***	-0.10 (-3.5)***	0.50
Lotus	UV-A	3.44 (13.8)***	-0.18 (-8.4)***	-0.09 (-3.2)**	0.50
	UV-B	3.22 (14.3)***	-0.17 (-8.7)***	-0.08 (-3.1)**	0.47
	Control	3.28 (14.6)***	-0.17 (-8.9)***	-0.08 (-3.2)**	0.47
Harvest Two					
Lolium	UV-A	0.92 (11.7)***	-0.05 (-7.1)***	-0.02 (-2.4)*	0.40
	UV-B	1.08 (11.0)***	-0.06 (-6.6)***	-0.03 (-2.9)**	0.50
	Control	0.80 (12.8)***	-0.04 (-7.9)***	-0.02 (-2.7)**	0.50
Lotus	UV-A	1.44 (13.2)***	-0.07 (-7.1)***	-0.06 (-5.1)***	0.86
	UV-B	0.41 (4.4)***	-0.02 (-2.2)*	-0.02 (-1.6)^{ns}	n/a
	Control	1.35 (13.7)***	-0.06 (-7.2)***	-0.05 (-4.9)***	0.83
Harvest Three					
Lolium	UV-A	0.68 (10.8)***	-0.04 (-6.7)***	-0.02 (-2.4)***	0.50
	UV-B	0.72 (11.7)***	-0.04 (-6.8)***	-0.02 (-2.9)**	0.50
	Control	0.65 (12.8)***	-0.03 (-7.7)***	-0.02 (-2.9)**	0.67
Lotus	UV-A	1.00 (10.2)***	-0.05 (-5.9)***	-0.03 (-2.9)**	0.60
	UV-B	0.25 (5.6)***	-0.01 (-1.6)^{ns}	-0.01 (-3.2)**	n/a
	Control	0.81 (12.7)***	-0.03 (-3.5)***	-0.04 (-7.5)***	1.33

The response-surface analysis therefore provides many conclusions when looked at its entirety. The competitive balance changed with time with the final two harvests showing similar results. In these later stages, it also appeared that UV-B had a detrimental effect on *Lotus* growth in particular. Most noticeably, the competitive effects were negated under UV-B stress.

5.3.3: *Aboveground Biomass Metabolic Fingerprinting*

Only the third harvest showed significant effects on the metabolome of both species (Figure 5.2). It was also apparent that UV-B altered the metabolome of leaves whilst there was no difference between control and UV-A conditions.

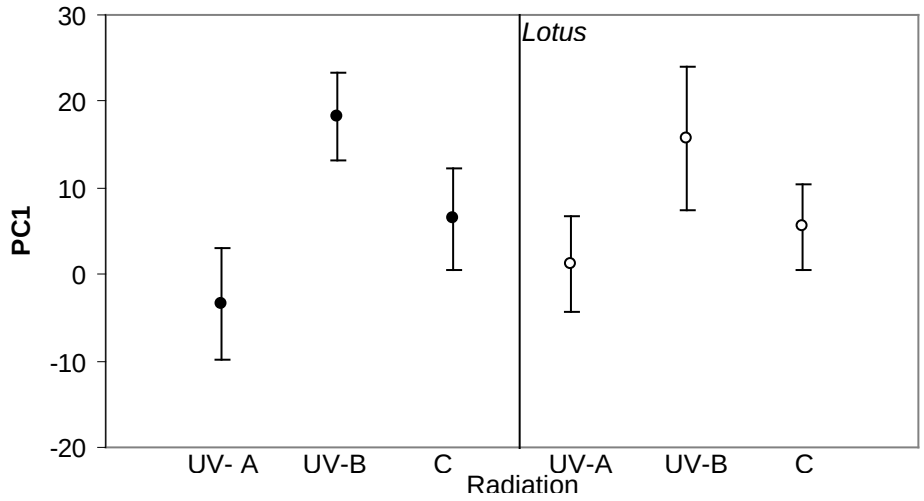


Figure 5.2. Effects of radiation on the metabolome of both *Lolium* and *Lotus*. Data is for the third harvest only as first two harvests showed no differences. For both species there was no difference between control and UV-A frames. No metabolic changes elicited by competition were observed.

5.3.4: *Soil Leachate Metabolic Fingerprinting*

Only the first harvest showed a significant effect on the soil leachates (Figure 5.3). There was an alteration in leachate profile with the shift from grass to legume dominated communities. The *Lotus* monoculture had the most observable differences however and also the greatest standard error. It is apparent that a linear relationship exists in the first harvest which suggests that community structure can be identified from the leachate profile. However, the different communities can only be identified for this experiment only as the methodology, as discussed in Chapter Three, is not reproducible for separate experiments.

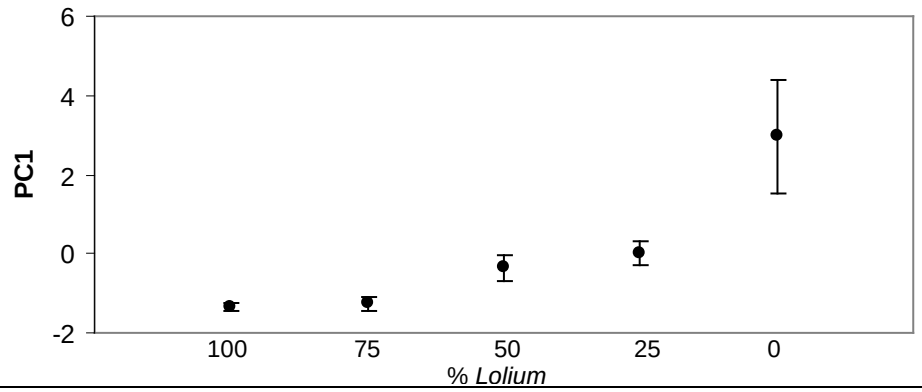


Figure 8.3. Alteration in soil leachate metabolome with shift in species composition for the first harvest. No pattern was observed for the second and third harvests. % *Lolium* is shown with remaining % being that of *Lotus*.

5.4: Discussion

5.4.1: *The Effect of UV-B on Plant Interactions*

Research that has focussed on the effects of UV-B on plant competition has been limited (Furness *et al.*, 2005b) and has centred on the ideas of Barnes *et al.* (1990b; 1995; 1996). Barnes *et al.* (1990b) originally hypothesised that UV-B would alter the canopy structure in mixtures of *Triticum aestivum* and *Avena fatua* which would consequently alter competition for light. This was substantiated by empirical work (Barnes *et al.*, 1995) that found the biomass of *T. aestivum* was unaffected although its fractional component to the leaf area index (LAI) was decreased by 5–7 % allowing an increase in light interception of 6-7 %. A similar conclusion was also drawn from studies using *Lycopersicon esculentum* and *Cucumis sativum* (Barnes *et al.*, 1996). Therefore, the most widely accepted hypothesis is that UV-B alters competition via shifts in canopy structure (Furness *et al.*, 2005b).

This theory is at variance with the results gained from this experiment. The theory of Barnes *et al.* (1990b) is only tenable for situations where there are only changes in morphology and not biomass. These results clearly show that UV-B alters biomass (Figure 5.1) and it is these changes that are responsible for changes in competition (Table 5.1). Moreover, small trial experiments were conducted at monthly intervals throughout the experiment (data not shown as ultimately of little relevance although all records kept) which showed that leaf area parameters, chlorophyll levels and dark respiration were not affected by UV-B in this experiment. In support of this, a Chinese study (Yuan *et al.*, 1999), using the same species as Barnes *et al.* (1995),

found that biomass changes were more responsible for competitive shifts than canopy structure. Furthermore, an early study suggested that differing susceptibility to UV-B was responsible for competitive ability (Fox & Caldwell, 1978). This is paralleled by the high susceptibility of *Lotus* to UV-B and the ability of *Lolium* to tolerate the stress (Table 5.1). However, another early study found that high levels of UV-B altered the interaction but not the biomass (Gold & Caldwell, 1983). Therefore, two contradicting hypotheses, each with supporting evidence, can be identified: UV-B mediated changes in competition are caused by (1) morphological changes or (2) biomass changes.

There have also been two notable studies that are of direct relevance to this experiment. Firstly, Norton *et al.* (1999) found that UV-B increased the proportion of *Lolium* spp. in an experimental grassland community. Exactly the same pattern occurred in this experiment as can be witnessed in the disparity in constant size between *Lolium* (1.08 g in Harvest Two; Table 5.1) and *Lotus* (0.41 g in Harvest Two; Table 5.1). Secondly, Furness *et al.* (2005a) used similar surface-response analysis to study the effects of UV-B on *Chenopodium album* and *Brassica oleracea*. Furness *et al.* (2005a) also found that UV-B lowered the substitution rate for *B. oleracea* (as with *Lotus* in this experiment) but increased the substitution rate for *C. album* (as with *Lolium* in this experiment). In both cases the species with the increased substitution rate due to UV-B (*C. album* and *Lolium*) predominated.

Despite research into UV-B and competition being limited, the importance of facilitation in ecology (Bruno *et al.*, 2003) and the need to understand the effects of stress on grass-legume interactions (Soussana & Lafarge, 1998) has resulted in

numerous studies investigating the effects of UV-B on legumes. Musil *et al.*, 2003 suggested that UV-B does not alter the biomass of legumes although alters biochemical characteristics which in turn affect competitive ability (Chimphango *et al.*, 2003a). Studies using tropical legumes (Chimphango *et al.*, 2003b; 2004) have substantiated this showing increased nodulation and flavonoid content of the roots with enhanced UV-B. A similar pattern was observed by Singh (1996; 1997). The results from this study refute the initial hypothesis that biomass changes are not present as they are clearly observed in Figure 5.1. It is therefore unlikely that all competitive shifts will be due to biochemical alterations alone. Nonetheless, analysis of the metabolome in *Lotus* leaves (Figure 5.2) shows that UV-B alters the biochemical profile and this may function in concert with biomass shifts to influence the outcome of competition.

5.4.2: *The Effect of UV-B on Soil Leachates*

The leachate collection of this experiment showed no alteration in leachate quality due to radiation. The only changes were found in the first harvest due to community structure (Figure 5.3). This is in accordance with Verhoef *et al.* (2000) who collected leachates from lysimeters filled with dune grassland soil from the Netherlands. As with this study, no changes were observed due to UV-B which is possibly because both studies were conducted outdoors using an inherently more variable system. The other studies were conducted under more controlled conditions where differences would be more noticeable.

Nonetheless, there have been a number of studies that have found UV-B mediated alterations in root exudation using natural communities. Avery *et al.* (2003) found that attenuating ambient UV-B altered rhizosphere microbial communities associated with *Deschampsia arctica*. A similar result was obtained from a study into the effects of enhanced UV-B on a disturbed upland grassland (Avery *et al.*, 2004) which suggested that altered root exudation was directly responsible. Moreover, by using dissolved organic carbon (DOC) and monocarboxylic acid as an estimate of root exudation, Rinnan *et al.* (2006) found altered exudation due to UV-B in two sub-Arctic mire species which was used as the basis to explain previously observed alterations in belowground microbial communities (Rinnan *et al.*, 2005). Rinnan *et al.* (2007) have hypothesised that root exudation could be key to understanding belowground studies. Whilst this study shows that changes to biomass cannot be ignored, it is clear that further research into root exudation is valuable.

Additionally, there has been further field-work investigating the effects of UV-B on belowground microbial communities which may play a role in competitive interactions (Johnson, 2003). Work on sub-Arctic heaths has shown shifts in belowground C:N ratio and bacterial communities (Johnson *et al.*, 2002) and formation of mycorrhizae in *Betula pendula* (De La Rosa *et al.*, 2003). Alterations in mycorrhizae have also been observed in a Dutch grassland (van de Staaij *et al.*, 2001) and an Argentinean fen (Zaller *et al.*, 2002). However, a study by Newsham *et al.* (1999) using *Quercus robur* from the British Isles found no such changes. Microbial research techniques such as the Biolog plates employed by Johnson *et al.* (2002) and genetic profiling using as DGGE (Kadiver & Stapleton, 2004) on similar mesocosms to the ones in this experiment would help elucidate belowground mechanisms further.

DGGE would be particularly useful as it provides a relatively rapid way to compare belowground microbial composition and would show how UV-B is altering the belowground biota (Arias *et al.*, 2005).

5.4.3: *UV-B Stress in the Tilman-Grime Debate*

As mentioned in the introduction to this chapter, there has been more discussion into the Tilman-Grime debate than actual tests of the theory. Research by Keddy *et al.* (1994; 1997; 2000; 2002) has been the most extensive research into empirically testing the theories. On balance the group has concluded, as with this study, that Grime's theory is possibly the most tenable. One study found that an index of competitive asymmetry (similar to substitution rates) declined with soil productivity (Keddy *et al.*, 1997) and another found that competition intensity was greatest in the most productive environments (Twolan-Strutt & Keddy, 1996). Fraser and Keddy (2005) also extended Grime's theory to suggest that at high soil fertilities (i.e. an absence of stress) the relative competitive ability of a species can predict the outcome of competition.

However, Fraser and Keddy (2005) also noted that there were exceptions. One study showed that the competitive response depended on environmental conditions although the competitive effect was constant across the environments (Keddy *et al.*, 1994). This is similar to the pattern seen for *Lotus* in the final two harvests (Table 5.1). Furthermore, in other experiments, it was found that there were no effects of competition under a variety of conditions along a shoreline (Keddy *et al.*, 2000) and along a soil depth gradient (Belcher *et al.*, 1995). Nonetheless, the balance of

evidence points towards a situation whereby abiotic stress negates competitive intensity.

Further tests have also been conducted that corroborate Grime's theory. Campbell and Grime (1992) concluded that their theory was substantiated from empirical observations. Turkington *et al.* (1993) found that competition was of little importance in disturbed areas which also supports Grime's theory. Peltzer *et al.* (1998) in a study originally designed to test Tilman's theory concluded that there was not sufficient evidence to support it. Moreover, many conservationists have applied Grime's theories (Harte & Shaw, 1995) to studies involving habitat management where grazing disturbance is present (Debain *et al.*, 2005; Kuijper *et al.*, 2005) and invasive species control (Dukes & Mooney, 1999). In the case of invasive species it has been suggested that an understanding of the competitive ability of weeds in relation of stress is of paramount importance in understanding weed control (Vila *et al.*, 2004; Hastwell & Panetta, 2005; Pan *et al.*, 2006).

Nonetheless, Tilman's theory has also been substantiated by previous studies. Moreover, even though it is clear that UV-B decreased the net effect of competition in this study, it did not eliminate the competitive response of *Lolium*. This suggests that some species may behave in a manner predicted by Tilman depending on circumstance. Paradoxically, Wilson and Tilman (1993) even believed that the results of Campbell and Grime's (1992) experiment had more evidence in support of their theory than Grime's. Theodose *et al.* (1996) found that in harsh alpine environments it was still possible for species to exhibit traits of competitors such as high resource uptake. Additionally, Theodose and Bowman (1997) found that it was possible under

such environments for one species to competitively displace another. Davis *et al.* (1998) explicitly claimed that Grime's theory could not explain the competitive dominance of tree species over grasses in a savannah. Rosch *et al.* (1997) came to a similar conclusion in a South African study. Ditommaso and Aarsen (1991) also concluded that Grime's theory was untenable using three European grassland species. Thus despite the evidence from this thesis which on balance supports Grime, there is still a substantial body of evidence which has proved Tilman's theory to be correct and this needs to be accounted for.

One possible explanation for the disparity in these studies is that UV-B is clearly a stress although unrelated to resource. Most studies, including all the ones in support of Tilman, used low resource levels in order to create conditions of stress. However, there have been a number of studies involving a stress that is not related to resource, namely salinity (Isacch *et al.*, 2006), and in all but one study (Costa *et al.*, 2003), they have shown the environment was key in structuring communities and not competition (Brewer *et al.*, 1998; Lenssen & De Kroon, 2005; Pennings *et al.*, 2005). La Peyre *et al.* (2001) found that stress-tolerators dominated and concluded that Grime's theory was the most tenable. This supports findings form this experiment where it was the species that most able to tolerate a non-resource-based stress (*Lolium*) that predominated.

There have been other theories that have attempted to resolve the fact that evidence exists for both theories. Goldberg & Novoplansky (1997) suggest the debate is essentially semantic; a sentiment echoed by Craine (2005) and Gaucherand *et al.* (2006). Goldberg *et al.* (1999) have even suggested a hypothesis that both theories

can coexist depending on ‘pulses’ of nutrient availability. It is therefore likely that a solution of the debate will arise from a greater awareness that both theories are tenable under certain circumstances. Whilst this opens up even more avenues of research it suggests that a synthesis of the debate is finally being realised.

5.4.5: *Conclusion*

In conclusion, the results tend to suggest that UV-B lowers the effects of competition via differential susceptibility of *Lolium* and *Lotus*. In terms of the Tilman-Grime debate this favours Grime. However, it cannot be said that Grime’s theory is the only possible mechanism as it is becoming increasingly likely that the two theories depend on circumstance.

5.4.6: *Summary*

1. *Lotus corniculatus* was highly susceptible to enhanced UV-B radiation whilst *Lolium perenne* was more tolerant resulting in communities dominated by the grass at the expense of the forb
2. In most circumstances, *Lotus corniculatus* competition coefficients were smallest under enhanced UV-B which supports Grime’s theory that competition decreases under stress
3. In one case, metabolic fingerprinting showed that the chemical profile of soil leachates reflected the plant community from which they were obtained.

Chapter Six: The use of non-nodulating *Lotus japonicus* mutants to ascertain the role of nitrogen fixation and UV-B in legume-grass facilitation

6.1: Introduction

Two main questions were raised from the previous two chapters. The first was whether the facilitative effect between *Lolium perenne* and *Lotus corniculatus* was due to nitrogen fixation as previously assumed. The second was whether the effect of UV-B on the interaction was partly due to indirect effects on belowground communities. In both cases, the role of nitrogen-fixation is called into question. *Lotus japonicus* mutants (Ljsym4), incapable of forming both mycorrhizal and nitrogen fixing associations, were therefore used to ascertain the extent belowground organisms have on plant interference. The following introduction outlines the two hypotheses that have been determined from previous work.

The first hypothesis is that *Lolium perenne* and *Lotus japonicus* will interact differently depending on the presence of nitrogen fixation. Grasses and legumes often exhibit a facilitative effect whereby combinations of the two are more productive than the two respective monocultures (Luscher et al., 1992; Connolly et al., 2001; Gosling, 2005). This has been attributed to the nitrogen fixing capacities of the legume which possibly enrich the surrounding environment to the benefit of all neighbouring species (Dupraz et al., 1998; Li et al., 2003; Rodriguez-Echeverria et al., 2003). This has been questioned previously in this thesis and non-nitrogen fixing mutants provide the opportunity to test this hypothesis further. If nitrogen fixation is directly responsible for facilitation as is widely believed (Hauggaard-Nielsen & Jensen, 2005; Høgh-

Jensen, 2006), then a mixture with a mutant that cannot fix nitrogen would not be expected to show facilitation. In this instance, the legume mutant should act as a competitor for the grass (Marquez & Allen, 1996).

The second hypothesis is that the effect of UV-B is dependent on whether nitrogen fixation occurs in the species mixture. UV-B has been shown to alter the quantity and quality of flavonoid compounds in exudates (Verhoef et al., 2000; Pinto et al., 2002) which are the same chemicals that determine belowground mycorrhizal communities (Siqueira *et al.*, 1991; Chabot *et al.*, 1992). It could be hypothesised that the bacteria responsible for nitrogen fixation will therefore be altered by UV-B which will then feedback into the plant interaction. By comparing ambient levels of UV-B and non-UV-B controls, alterations to competitive interactions could be expected due to alterations in symbiotic relationships.

6.1.3: *Aims*

1. Test the hypothesis that the facilitative effect is due to nitrogen fixation
2. Test the hypothesis that belowground microbial associations mediate the effect of UV-B
3. Assess the potential of FT-IR in detecting shifts in plant interference

6.2: Materials & Methods

6.2.1: *Plant Communities*

The experiment consisted of three plant interactions: (1) *Lolium perenne* and *Lotus japonicus*; (2) *Lolium perenne* and *Lotus japonicus* Ljsym4 mutant; (3) *Lotus japonicus* and *Lotus japonicus* Ljsym4 mutant. Each interaction was designed and analysed using the response surface design (as explained in the plant interference methodology section). Each mixture consisted of ten communities with two densities (five pots with four plants per pot and five pots with twelve plants per pot) and five relative frequencies (percentage ratio of first species to second species: 100:0; 75:25; 50:50; 25:75; 0:100). There were therefore thirty communities per replicate (three interactions and ten communities). This was replicated four times in two growth cabinets representing attenuated UV-B and ambient levels of UV-B (parameters the same as those in methodological development section). There were therefore 240 experimental units (two levels of UV-B x 30 communities x 4 replicates).

Seeds of *Lolium perenne* were obtained from Chiltern Seeds (Chiltern Seeds Ltd., Ulverston, Cumbria, UK) whilst *Lotus japonicus* Ljsym4 mutants and *Lotus japonicus* 'Gifu' wild-type were obtained from IGER (Aberystwyth, Ceredigion, UK) courtesy of Judith Webb. Each community was sown directly into a cell of a 26 cm x 36 cm green-plastic tray divided into twenty cells (John Innes No. 3). After one-week any extraneous seedlings were removed and communities left for another week before moving to the growth cabinets. The seedlings were left for eight weeks in the growth cabinet before all above-ground biomass was harvested and dried at 60°C prior to

weighing. The biomass was used as the yield response in the response surface analysis. Samples from dried material were also used for metabolic fingerprinting.

6.3: Results

Nodulation could be observed on all the wild type *Lotus* species. Growth in the mutant wild type was very slow and the roots system was particularly weak (typically just three roots no longer than 5 cm).

6.3.1: *Community Biomass*

There was no facilitative effect observed between the grass and wild-type legume (Figure 6.1). The monoculture biomass for *Lotus* was smaller as could be expected from a facilitative effect although the *Lolium* monoculture was greater than the mixtures. The same pattern was observed for *Lolium* and mutant *Lotus* mixtures although the disparity between the two monocultures was pronounced (Figure 6.1) suggesting that *Lotus* mutants grew more slowly than their wild-type counterparts. All the mixtures of wild-type *Lotus* and mutant *Lotus* were similar.

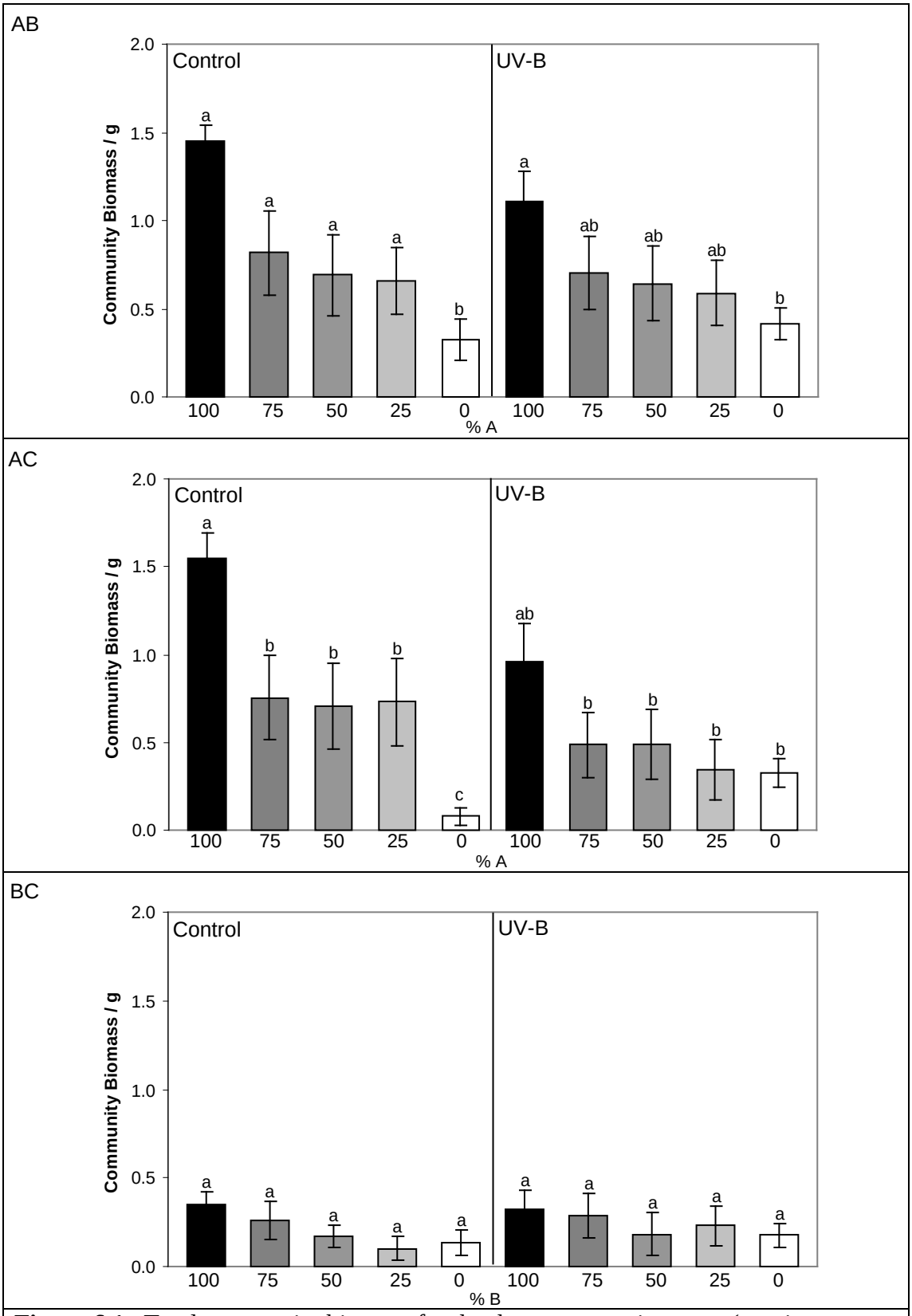


Figure 6.1. Total community biomass for the three community types (species mixture in top-left corner: *Lolium* – A; *Lotus* wt – B; *Lotus* mutant – C) and both UV-B and control treatments. % of one species given (the two densities combined). Different letters denoted statistical differences ($p<0.001$)

The biomass yield of *Lolium* and wild-type *Lotus* under UV-B was similar to the control communities. The same also applied to mixtures of *Lolium* and Ljsym4 mutants suggesting this mixture acts similarly to non-mutant mixtures in UV-B. The mutant *Lotus* monoculture was greater under UV-B than it was in the control ($P<0.001$) suggesting that ambient levels of UV-B could encourage growth of mutants whilst not affecting the wild-type. There was no effect of UV-B in the *Lotus* wild-type and mutant mixture with all combinations possessing a similar biomass to the control.

6.3.2: *Species Interference*

The intra-specific effect was always greater than the inter-specific effect in mixtures of grasses and nitrogen-fixing legumes as can be seen by the greater coefficients in Table 6.1. The constants for *Lolium* were always greater than *Lotus* as could be expected from the larger monocultures (Figure 6.1). Under UV-B, the *Lolium* coefficients decreased although this was commensurate with the lower constants (and hence overall biomass) which suggests that UV-B is not directly altering competition. However, for *Lotus*, there were no significant coefficients under UV-B suggesting that competition is completely removed under stress.

Table 6.1. Competition coefficients for the three species (Sp.) (*Lolium* – A; *Lotus* wt – B; *Lotus* mutant – C). Companion species (Comp.) shares similar notation and radiation (Rad.) is divided according to elevated UV (UV) or control conditions (C). T-values are shown in parentheses along with significance.

Sp.	Comp.	Rad.	Constant	Intra-specific	Inter-specific
A	B	C	0.97 (10.8)***	-0.08 (-7.1)***	-0.03 (-2.3)*
		UV	0.83 (10.6)***	-0.07 (-7.1)***	-0.02 (-1.7)^{ns}
	C	C	1.04 (9.9)***	-0.09 (-6.7)***	-0.03 (-1.7)^{ns}
		UV	0.65 (5.8)***	-0.06 (-3.8)***	-0.02 (-1.5)^{ns}
B	A	C	0.16 (5.1)***	-0.01 (-3.4)**	-0.01 (-1.4)^{ns}
		UV	0.12 (2.7)*	-0.01 (-1.6)^{ns}	-0.01 (-0.7)^{ns}
	C	C	0.21 (5.3)***	-0.02 (-2.9)**	-0.01 (-2.1)*
		UV	0.27 (4.3)***	-0.02 (-2.9)**	-0.00 (-0.4)^{ns}
C	A	C	0.05 (2.0)^{ns}	-0.01 (-1.5)^{ns}	0.00 (0.3)^{ns}
		UV	0.17 (2.6)*	-0.02 (-1.8)^{ns}	-0.01 (-0.8)^{ns}
	B	C	0.06 (2.7)*	-0.00 (-1.5)^{ns}	-0.00 (-1.2)^{ns}
		UV	0.05 (3.1)**	-0.00 (-1.4)^{ns}	-0.01 (-2.3)*

A similar pattern was observed for mixtures of grasses and mutants with intra-specific coefficients being greater than inter-specific coefficients for both species. The constants for the wild-type *Lotus* were significantly higher (0.97; Table 6.1) than the mutants (0.05; Table 6.1) which again illustrates the difference in size between wild-types and mutants. The *Lolium* constant and coefficient decreased under UV-B as with the wild-type mixtures which corroborates the pattern seen in the other mixture. Moreover, the mutant legume behaved like the wild-type and there were no differences due to UV-B suggesting *Lotus* did not compete. Competition had no effect in the two-legume mixture with the only marginally significant coefficient being a noticeably low -0.01 (Table 6.1).

6.3.3: *Metabolic Fingerprinting*

An analysis of the metabolome for each species, regardless of relative frequency, shows that the companion species has a significant effect on the metabolome. For example, *Lolium* (Figure 6.2a) had a different profile depending on whether it had been grown amongst wild-type *Lotus* or Ljsym4 mutants. Similarly, wild-type *Lotus* has a different profile depending on whether it has been grown with *Lolium* or Ljsym4 mutants. Importantly, the same effect applied to *Lotus* mutants (compare Figure 6.2b and 6.2c). This firstly suggests that the biochemistry of the two species is very similar. Secondly, it suggests that a form of intra-specific competition (the other *Lotus* species) can be discerned from the inter-specific competition.

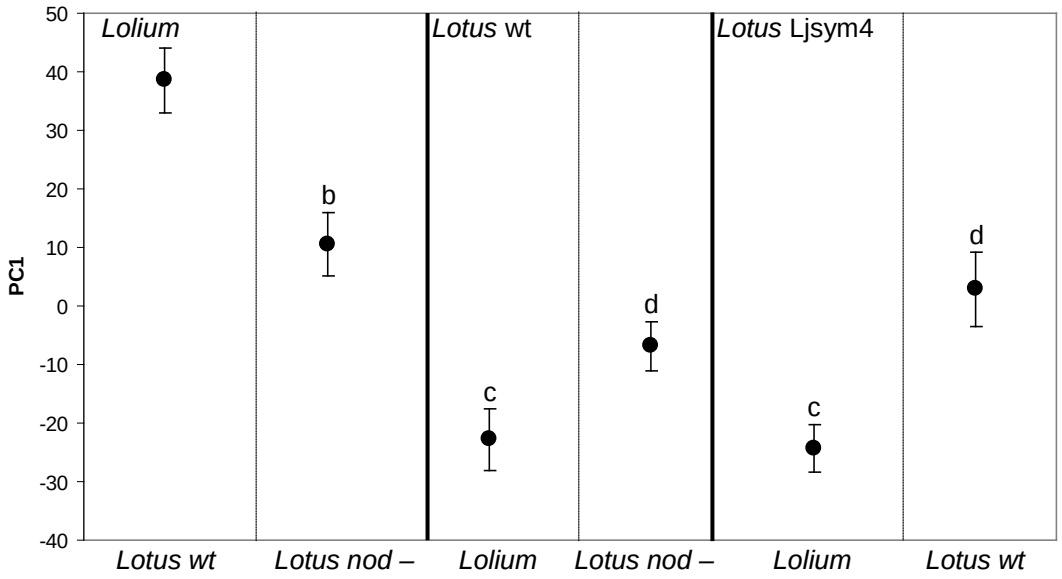


Figure 6.2. Effect of companion species on metabolome of the three species used. The top line shows the target species whilst the bottom line shows the two possible companion species.

Figure 6.3 shows how the metabolome of an individual shifts with increasing relative frequency. Figures 6.3b and 6.3c show that the metabolome becomes more

convergent at higher densities for the two *Lotus* strains whilst Figure 6.3a suggests that the metabolome of *Lolium* is more affected by an increase in the other species. The shifts in the metabolome suggest that the type of competition and its intensity could be inferred by further metabolomic analysis.

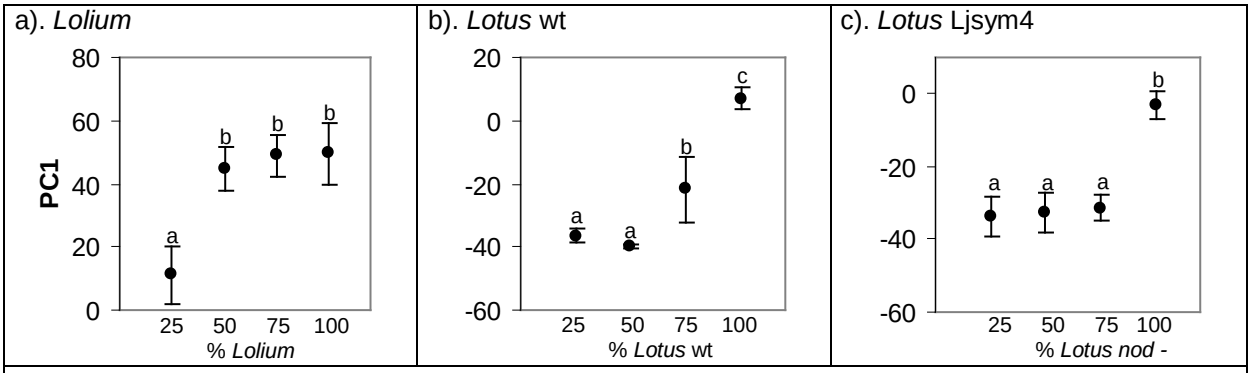


Figure 6.3. Alterations in metabolome for *Lolium* (a), *Lotus* wt (b) and *Lotus*Ljsym4 (c) with an increasing respective proportion of companion species (labelled on lower axis)

6.4: Discussion

6.4.1: *The Role of Nitrogen Fixation in Plant Interference*

The first hypothesis was that wild-type grass-legume mixtures would overyield whilst mixtures of grasses and non-fixing legume mutant legumes would not. This hypothesis can be initially rejected as facilitation did not occur for either mixture (Figure 6.1). Despite the *Lotus* monocultures being less productive than the two-species mixtures, as can be expected from a facilitative effect, the *Lolium* monocultures were the most productive (Figure 6.1). Nonetheless, if nitrogen-fixation was still important in determining the biomass of grass-legume mixtures then it could be hypothesised that the mixtures would still be different between wild-type and mutant mixtures. However, this was not observed (Figure 6.1).

This observation supports evidence from this thesis that suggests facilitation is not a result of nitrogen-fixation in the early stages of competition but a consequence of decreased intra-specific competition. Results from the grass-legume optimisation experiments and enhanced UV-B experiment all showed that intra-specific competition was more intense than inter-specific resulting in facilitation due to lowered competition intensity. This hypothesis has been supported by other studies that have questioned the traditional view that nitrogen-fixation is the basis of over-yielding (Høgh-Jensen, 2006). It has become apparent that not all legumes show a facilitative effect (Marquez & Allen, 1996) whilst facilitation is not only observed in mixtures containing legumes (van Ruijven & Berendse, 2003). Furthermore, it is believed that facilitation could be observed in all species mixtures due to alteration in competition mechanisms (Hooper & Dukes, 2004). It is therefore possible that there would be no difference between wild-type and mutant grass-legume mixtures, as observed in this study, should mechanisms other than nitrogen-fixation account for differences in community structure.

6.4.2: *The Effects of UV-B*

The second hypothesis was that ambient levels of UV-B would alter the competitive balance between grass-legume mixtures whilst it would not in mixtures of grass and non-fixing legume mutants. UV-B had no effect on the wild-type mixtures and the only effect on the mutants was an increase in biomass of the Ljsym4 mutant monoculture. However, as this was the only UV-B mediated difference, the results suggest that ambient levels of UV-B are not sufficient to elicit noticeable differences

in the mixtures used. This has been shown in other studies where UV-B did not dramatically affect plant biomass in competitive interactions (Barnes *et al.*, 1995; 1996). Moreover, even enhanced levels of UV-B have only elicited a small change in natural communities (Björn *et al.*, 1997). The evidence from this study therefore supports the common view that doses of UV-B similar to ambient have little effect on plant interactions.

The fact that UV-B did not alter the interaction between *Lolium* and Ljsym4 mutants provides further support that belowground interactions may not always determine the aboveground competitive response. It could be hypothesised that UV-B would alter the flavonoid composition of the plants (Shirley, 1996) which would in turn affect the chemical composition of the root exudates (Klironomos & Allen, 1995). This would then alter the microbial communities (Shiozaki *et al.*, 1999; Pinto *et al.*, 2002) which would have consequences for plant growth. As UV-B did not affect the competitive effect in wild-type mixtures compared to mutant mixtures, this hypothesis can be questioned. At first glance, this initially conflicts with the growing evidence that UV-B exerts a greater effect belowground than aboveground (Johnson *et al.*, 2002). There is a widely-held consensus that the commonly observed belowground alterations are plant-mediated (Rinnan *et al.*, 2007) given UV-B cannot directly penetrate the soil (Green, 1983). However, there is no reason why belowground parameters could change (which was also possible in this experiment) without eliciting an aboveground shift. For example, the substantial belowground changes in the sub-Arctic (Johnson, 2003) are evidently not reflected in the aboveground heath (Phoenix *et al.*, 2000).

6.4.3: *Conclusion*

By testing the hypothesis that facilitative interactions would not occur in mixtures of grasses with non-fixing legumes, it has been shown that the role of nitrogen fixation has possibly been exaggerated in plant interference. Instead, these results corroborate previous experiments that have shown that facilitation is a result of decreased intra-specific competition. Ambient levels of UV-B were shown to have little effect on the interaction.

Future work should include a longer time-scale; especially given root-exudate quality has been shown to alter over time (Warembourg & Estelrich, 2001). The introduction of the experiment to an open system or outdoor site would be of particular benefit given that different microbial communities can influence the aboveground system in different ways (Heijden *et al.*, 2006). However, consideration would have to be given to the use of genetic mutants in the open environment. Nonetheless, this study has shown that the use of mutants in plant interference is informative and should be more widely employed in similar studies.

6.4.4: *Summary*

1. *Lotus corniculatus* var. *japonicus* mutants that cannot fix nitrogen are highly effective tools in assessing the role nitrogen fixation plays in plant interactions
2. The results confirm the results from the previous sections that nitrogen-fixation plays a small role in the interaction in the early stages of competition
3. The ambient levels of UV-B in the growth cabinet did not significantly affect the competitive interaction

Chapter Seven: The Interaction Between UV-B and Nutrient Availability in an Artificial Sub-Montane Community

7.1: Introduction

Chapter Five showed that enhanced levels of UV-B (as opposed to ambient levels in Chapter Six) was important in altering the competitive interaction in an artificial grass-legume assemblage. In order for such studies to be made more ecologically relevant it is important to firstly extend the complexity of the plant interactions by having more than two species and secondly to extend the complexity of the environmental perturbation (for example, enhanced UV-B is unlikely to be the only abiotic stress). This chapter presents an experiment that incorporated two abiotic factors (UV-B and nutrient availability) on a competitive interaction involving three species commonly found in sub-montane communities (*Agrostis tenuis*, *Festuca ovina* and *Galium saxatile*). The introduction initially argues the reason for using nutrient availability as a second abiotic factor on the grounds that nitrogen-deposition pollution is an increasingly common disturbance. The susceptibility of sub-montane communities is then presented before introducing an hypothesis that suggests that the extent of the UV-B effect is dependant on nutrient availability.

7.1.1: *Nitrogen Deposition and Plant Interactions*

All life exists in an atmosphere dominated by nitrogen. More than three-quarters of ambient air comprises of dinitrogen gas (N_2) with the oxygen and carbon dioxide present in lower quantities. Nitrogen gas is not required for most biological processes

although nitrogen itself is a crucial component of many organic molecules; notably proteins – the building blocks of life. However, despite its abundance and necessity, only a small amount of nitrogen gas is naturally fixed (predominantly through lightning and bacterial fixation) into compounds that can be utilised by biological systems (Jordan & Weller, 1996). Thus notwithstanding its ubiquity, many biological systems are limited by nitrogen present in usable forms.

The conversion of dinitrogen gas to usable reactive compounds (commonly denoted by Nr) has been vastly altered by anthropogenic processes. More nitrogen is fixed by humans than by all natural processes combined (Vitousek, 1994). Prospero *et al*. (1996) calculated that human-based nitrogen fixation is four to five times greater than natural processes. In one of the earliest studies outlining the problem, Hamed & Dignan (1992) suggested that global nitrous oxide (NO_X) emissions rose from 18 Mt to 24 Mt between 1970 and 1986. Galloway *et al*. (1995), in the most widely cited review on this subject, predicted that anthropogenic nitrogen fixation will increase 25 % by 2020 (when the human population is predicted to be 8.5 billion) with the greatest fixation occurring from South-East Asia and South America. With up to 60 % of the artificial usable nitrogen retained by natural systems (Lee, 1998), it is clear that humans have substantially altered the global nitrogen cycle.

Changes to agricultural practice are the predominant reason why anthropogenic nitrogen fixation has altered so much. 57 % of the increased nitrogen fixation is from artificial fertiliser with a further 29 % from the widespread cultivation of nitrogen-fixing legumes for fodder (Galloway *et al*., 1995). Prospero *et al*. (1996) suggested that increased farming of livestock was increasing nitrogen fixation by up to 10 %

from urea alone. Fertiliser use is set to increase rapidly as developing countries increasing demand of inorganic fertilisers (Bouwman *et al*., 2005) with approximately one third of all inorganic fertiliser being used in South-East Asia (Matthews, 1994). Galloway *et al*. (1996) noted that 80 % of all nitrogen compounds produced in China was from fertiliser compared to 50 % in the USA, although with increased industrialisation the net nitrogen production in China will increase. Thus the development of fertiliser cannot be underestimated in understanding the causes of why the global nitrogen balance has been dramatically altered. The fact that humans have developed a system to convert dinitrogen gas to biologically usable nitrogen compounds (fertiliser) has substantially altered the nitrogen cycle.

Whilst the overwhelming majority of anthropogenically fixed nitrogen is from agricultural practice, a substantial amount (14 %) comes from industrial processes (Galloway et al., 2005). The burning of fossil fuels releases nitrous oxides (NO and NO_2; commonly denoted as NO_X) which can in turn convert dinitrogen gas to more NO_X in the process (Norby, 1995). Under certain environmental conditions, these gases can be deposited as NO_3^- and NH_4^+ ions which can be taken up by plants (van der Eerden, 1998). The gases can also be absorbed through the stomata and directly incorporated into physiological processes although usable nitrogen compounds absorbed in such a way is small compared to uptake from the roots (Stulen *et al*., 1998). One key issue surrounding nitrogen deposition is that emissions can travel over long distances. Holland *et al*. (1995) suggested that nitrogen deposition from European emissions is likely to remain on the continent although pollution arriving from North America may increase nitrogen deposition further. Whilst the increase in reactive nitrogen from the burning of fossil fuels is not as great as that from fertiliser,

it is still widely studied. This is essentially as natural ecosystems are more likely to be affected by nitrogen deposition from pollution than fertiliser.

Therefore it is highly likely that increased pollution will have a significant effect on ecosystem functioning with the general consensus being that nitrogen deposition is likely to be damaging to the environment. Nadelhoffer (2000) studied the effects of nitrogen deposition in the context of the nitrogen saturation hypotheses and suggested that ecosystems will move from being nitrogen-limited to nitrogen-unbalanced (whereby nitrogen deposition alters ecosystem structure). Lee (1998) argued that the alteration in C:N ratio will increase carbon sequestration. Bobbink *et al*. (1998a) also listed a range of effects such as acidification of the soil, susceptibility of plants to herbivory and competitive exclusion in favour of nitrophilous species.

7.1.2: *Nitrogen Deposition and Sub-Montane Communities*

In the British Isles, the study of nitrogen deposition on natural communities has focused predominantly on upland sub-montane communities and heaths (Lee, 1998). Such communities are seen to be susceptible to nitrogen deposition as they have evolved under poor-nutrient conditions and are usually nitrogen limited (Bobbink *et al*., 1998a). The addition of nitrogen from pollutants may therefore shift the community structure to one of a nitrophilous structure. Thus research into the effects of pollution on these communities has practicable benefits to conservationists wishing to preserve the upland habitat.

Sub-montane communities are seen to be of particular interest to conservation as the uplands represent the most extensive semi-natural habitat of the British Isles. Around 6.5 Mha (28 % of the British Isles) is comprised of this habitat (Williams, 2006). The habitats are also of international importance as the long-term influence of humans along the Western Atlantic has resulted in an unique habitat found nowhere else on the globe (Ratcliffe & Thompson, 1988).

Of all the upland communities studied by ecologists, community U4 of the NVC system has received a large share of the focus (Hulme *et al*., 1999; Milne *et al*., 2002). U4 is characterised by base-poor soil whose nutrient content is kept low by constant leaching from heavy rainfall (Rodwell, 1993). Under such conditions it could be expected that nitrogen deposition will have a substantial effect on the nitrogen balance. The three key species are *Agrostis capillaris*, *Festuca ovina* and *Galium saxatile*. Other common grasses include the fescues *F. rubra*, *F. vivipara* and *F. tercifolia*. Herbaceous species are not particularly common as U4 is particularly species-poor although, other than the ubiquitous *G. saxatile*, it is common to find *Potentilla erecta*. Given the importance of U4 and its potential as a model community for sub-montane habitats, this project will focus on an artificial assemblage of the three key species from this community.

7.1.3: *The Interaction Between UV-B and Nutrient Availability*

It has been suggested that the extent of the effect of UV-B is dependent on nutrient availability (Correia *et al.*, 2005). It has been shown that the effects of UV-B are exacerbated by an increase in nutrient availability with plants grown under nutrient

limited conditions exhibiting greater tolerance of UV-B stress (Levizou & Manetas, 2001; Tosserams *et al*., 2001; Yao & Liu, 2007). The reasons for this effect are still debated (Correia *et al.*, 2005) although it can be hypothesised that plants grown in an excess of inorganic nitrogen, for example due to nitrogen deposition, will be negatively affected by UV-B. Given that current climate change scenarios predict an increase in nitrogen pollution (Galloway *et al.*, 1995) it is possible that the impact of enhanced UV-B may be greater than originally predicted. This chapter aims to test this hypothesis by studying the interaction between UV-B and nutrient availability on an artificial sub-montane community.

7.1.4: *Aims*

1. Test the hypothesis that the extent of UV-B effect is dependent on nutrient availability
2. Assess whether increased UV-B and nutrient availability alters the competitive balance
3. Assess the suitability of response surface designs to multi-species mixtures

7.2: Materials and Methods

7.2.1: *Experimental Set-Up*

A multi-factorial experiment was set-up to study the effects of ambient UV-B levels and different soil fertilities on the interaction between three species typical of a sub-montane U4 habitat. The three species used, *Agrostis tenuis*, *Festuca ovina* and *Galium saxatile*, were obtained from Chiltern Seeds (Chiltern Seeds Ltd., Ulverston, UK). A surface-response model was used to study the plant interactions. Twelve community combinations were chosen (represented in Figure 7.1). Half of the communities possessed a low overall density (six plants per pot) and the other half represented a dense sward (twelve plants per pot). For each density, each species was represented at low, medium and high relative frequencies. For example, there were two communities with one *Agrostis* per pot, another two with two *Agrostis* plants per pot and another two pots with four *Agrostis* specimens. The other two species were present at the other relative frequencies. For example, in the two pots with one *Agrostis* specimen, one possessed two *Galium* and four *Festuca* whilst the other possessed four *Galium* and two *Festuca*. The net effect was that each pot contained three relative frequencies with a different species at each frequency.

Three levels of soil fertility were used. The fertility of the soil was based on the fertiliser levels formulated using John Innes standard mixtures (where 1 is the lowest and 3 the highest for general horticultural use). These fertility levels were used as they provide a relative basis to see the effects of increasing fertility on plants. A drawback of using John Innes as basis for soil fertilities was it lacked ecological

relevance, especially with regards to nitrogen deposition. However, John Innes soils are widely used as a general standard for different fertilities (especially with regards to seedling stage) and were used in this experiment as the central concern was on the interaction of soil fertility level with UV-B. Therefore, whilst the high nitrogen levels could potentially be used to predict the effects of nitrogen deposition, it should be borne in mind during the discussion that John Innes bases are not sufficient to make precise models into the effects of nitrogen deposition. Throughout the discussion the levels of soil are denoted high, medium and low for relative ease of use.

Base soil was formed by mixing loam, peat and sand at a ratio of 7:3:2 respectively. The source of these soils could not be traced to obtain exact nitrogen release, although as mentioned above, the different levels of soil fertility were essentially to find a relative effect of UV-B. Furthermore, the base soil was the same for each mixture. Medium and high nutrient conditions were created by adding 226 and 452 grams of John Innes formula base fertiliser (see below for components) to 246 litres of base soil (this represents John Innes potting compost formulae 2 and 3+1 respectively). A control level was formed by using base soil with no added fertiliser. John Innes base fertiliser was created by mixing ammonioum nitrate fertiliser, phosphorus pentoxide (P_2O_5) and potassium oxide (K_2O) at a ratio of 5.1:8.2:10.0 respectively. The percentage (by weight) of nitrogen (N), phosphorus (P) and potassium (K) in the fertiliser is 3.5%, 3.6% and 8.3% respectively (NPK in 100g: 0.25 mol: 0.12 mol: 0.21 mol or 1 mol: 0.46 mol: 0.85 mol).

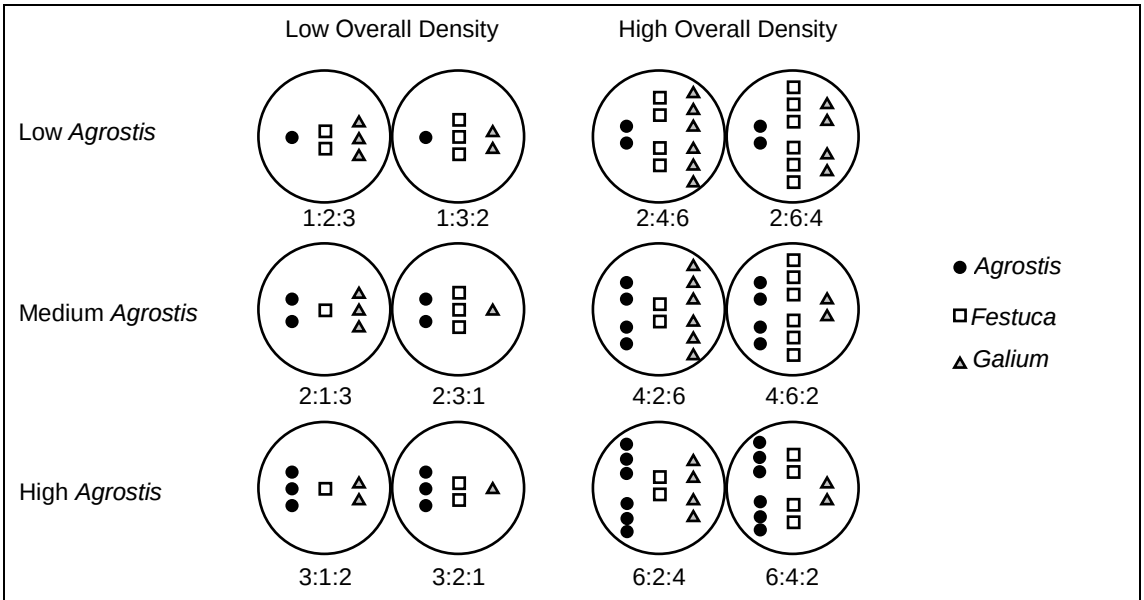


Figure 7.1. Schematic representation of the twelve communities used.

UV-B treatment was administered by using the two growth cabinets described in the methodological development chapter. One cabinet lacked UV-B and the other represented 8.3 $KJ/m^2/hr^{-1}$ which is similar to the maximum daily UV-B strength in June in Aberystwyth on a clear day. There were therefore 72 pots per complete complement of treatments (12 communities x 3 soil level x 2 radiation levels). These were replicated three times for a total of 216 experimental units.

The seeds were sown directly in the pots into the various treatment combinations under glasshouse conditions. Any excess seedlings were pricked out after ten days when all germination was complete. After four days the plants were then taken to the growth cabinets as it has been noted from previous experiments that germination was hindered in the growth cabinets (the reasons could be due to the quick drying of the soil due to the air circulation from the fans). The plants were then kept for ten weeks in the growth cabinets under conditions of 28°C with a photoperiod of 16 hours (total UV-B dose of 132.8 $KJ/m^2/hr^{-1}$ per day). Plants were watered twice a day. After ten

weeks the plants were then harvested above-ground and the organs dried at 60°C for 36 hours before being weighed.

7.2.2: *Statistical Analysis*

The statistical analysis of response surface designs has been elaborated on earlier in this thesis and the central precepts are essentially the same. However, this experiment is unique in that it includes three species instead of the standard two-species mixtures favoured by plant interference researchers. This poses little problem as another term is added to the basic format so that the individual of one species is affected by other individuals of the same species, individuals of another species and individuals of another species again. A standard formula would be:

$$W_X = c + C_X d_X + C_Y d_Y + C_Z d_Z \quad (1)$$

Where W_X represents the biomass of an individual of species X (in grams) and c the constant dX represents the density of species X in a pot and C_X the coefficient (effectively the number of grams lost or gained by each additional individual of species X). Y and Z represent the other two species. A total of eighteen models were analysed (3 species x 3 soil levels x 2 radiation levels) to assess how UV-B and soil nutrients alter the competitive balance.

7.3: Results

7.3.1: *Community Biomass*

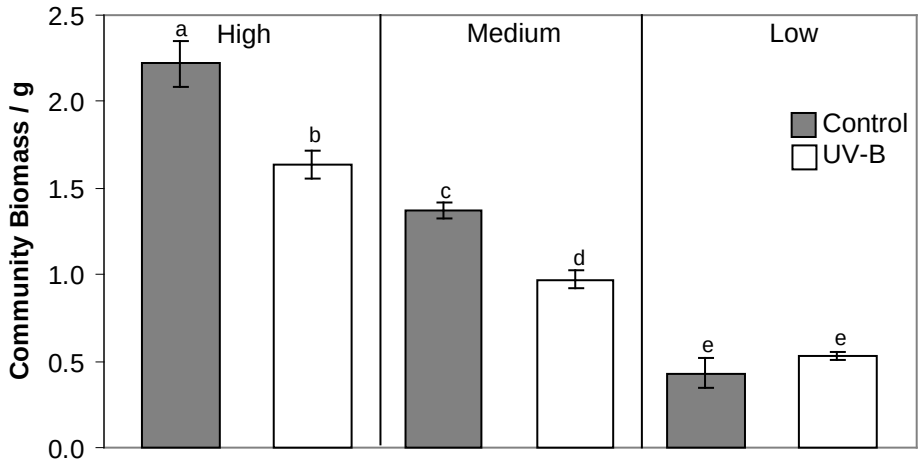


Figure 7.2. Figure showing the total community biomass as affected by nutritional quality of the soil (high – additional 0.03g nitrogen per litre, medium – additional 0.03 g nitrogen per litre and low status (no additional fertiliser) are shown at top of each column) and presence of UV-B (control, shaded; UV-B, blank).

The species composition of the communities had no effect on the total community biomass regardless of treatment ($F=1.03$; $P=0.401$). Therefore, the total biomass of a community was determined by abiotic factors as opposed to species composition.

Nutrient availability was highly significant ($F=173.17$; $P<0.001$) with an average biomass decrease of 65 % for each decrease in soil fertility level (Figure 7.2). UV-B also had a significantly detrimental effect on total community biomass ($F=21.08$; $P<0.001$) with a decrease of 35 % in high nutrient conditions and 41 % in medium nutrient conditions. There was a significant interaction between UV-B and nutrient

availability ($F=10.14$; $P<0.001$) as UV-B had no effect on community biomass under low nutrient conditions.

7.3.2: *Species Competition*

Only nine of the 18 possible models (3 species x 3 soil levels x 2 radiation levels) possessed significant competition coefficients (Table 7.1). This suggests that competition does not play a dominant role in shaping the relative proportions of species in the community.

Five of the nine models containing significant competition coefficients related to the competitive response of *Agrostis*. All five cases were intraspecific. The only model containing no significant differences for *Agrostis* was under low nutrient conditions in the absence of UV-B. The only other species to affect *Agrostis* was *Galium* which had a negative effect under conditions of medium soil fertility and UV-B.

The ratio of the *Agrostis* constant to the intraspecific coefficient (in effect, the maximum number of plants that can be grown together) was similar for all treatments. This suggests the intensity of the competition was similar regardless of the level of stress.

Table 7.1. Table showing competition coefficients. Abbreviations as follows: Rad., radiation; C, control; U, UV-B; Nut., nutrient level; H, high; M, medium; L, low; d, density; A, *Agrostis*; F, *Festuca*; G, *Galium*.

Rad.	Nut.	Constant	dA	dF	dG
Agrostis					
C	H	1.17 (6.6)***	-0.14 (-4.0)***	-0.05 (-1.4)^{ns}	0.01 (0.3)^{ns}
	M	0.60 (8.4)***	-0.07 (-5.2)***	-0.01(-0.9)^{ns}	-0.02 (-1.5)^{ns}
	L	0.01 (2.2) *	-0.05 (-1.0)^{ns}	0.00 (0.2)^{ns}	0.01 (1.2)^{ns}
U	H	1.32 (8.2)***	-0.17 (-5.5)***	-0.03 (-0.9)^{ns}	-0.03 (-1.0)^{ns}
	M	0.59 (6.7)***	-0.07 (-4.0)***	-0.01 (-0.3)^{ns}	-0.04 (-2.5)*
	L	0.22 (4.9)***	-0.03 (-3.0)**	-0.01 (-0.4)^{ns}	-0.00 (-0.4)^{ns}
Festuca					
C	H	0.17 (5.5)***	-0.02 (-3.2)**	-0.00 (-0.3)^{ns}	-0.00 (-0.1)^{ns}
	M	0.17 (7.1)***	-0.01 (-1.4)^{ns}	-0.01 (-2.0)^{ns}	-0.01 (-2.4)*
	L	0.06 (4.2)***	-0.00 (-1.1)^{ns}	-0.00 (-0.6)^{ns}	-0.00 (-1.4)^{ns}
U	H	0.04 (2.1)*	-0.01 (-0.4)^{ns}	-0.00 (-0.1)^{ns}	-0.01 (-0.3)^{ns}
	M	0.07 (2.9)**	-0.00 (-0.8)^{ns}	-0.01 (-1.0)^{ns}	-0.00 (-0.4)^{ns}
	L	0.14 (0.1)***	-0.00 (-0.9)^{ns}	-0.02 (-5.1)***	-0.00 (-1.1)^{ns}
Galium					
C	H	0.56 (1.8)^{ns}	0.00 (0.0)^{ns}	0.01 (-0.8)^{ns}	0.01 (-1.1)^{ns}
	M	0.44 (7.8)***	-0.03 (-2.3)*	-0.02 (-1.8)^{ns}	-0.04 (-3.3)**
	L	0.31 (2.21)*	0.00 (0.3)^{ns}	-0.00 (-1.2)^{ns}	-0.00 (-0.7)^{ns}
U	H	0.01 (1.8)^{ns}	-0.00 (-1.4)^{ns}	-0.00 (-1.4)^{ns}	0.00 (1.2)^{ns}
	M	0.02 (4.5)***	-0.00 (-1.3)^{ns}	0.00 (0.5)^{ns}	0.00 (-1.6)^{ns}
	L	0.03 (2.3)*	0.00 (1.2)^{ns}	0.00 (0.3)^{ns}	-0.00 (-1.1)^{ns}

Agrostis also had a negative effect on *Festuca* under high nutrient availability and no UV-B (a loss of 0.02g per individual) and *Galium* under medium nutrient conditions and no UV-B (a loss of 0.03g per individual). The only other significant coefficient related to the intraspecific competition between *Festuca* under low nutrient conditions and UV-B (a loss of 0.02 g per individual).

Agrostis also possessed the most biomass and thus constituted the largest proportion, in terms of biomass, in the mixtures. This can be observed by looking at the constants in Table 4.2 which effectively are the theoretical biomasses for individuals grown in the absence of competition. Under high nutrient conditions and the absence of UV-B, the biomass of an *Agrostis* individual was 52% greater than *Galium* and 85% greater than *Festuca*. This suggested that under high nutrient conditions *Agrostis* will possess the greatest biomass per species in the community.

7.3.3: *Metabolic Fingerprinting of Leaf Material*

UV-B had a significant effect on the metabolome of *Agrostis* under all three nutrient availability levels ($F=20.43$; $P<0.001$) (Figure 7.3). However, the metabolic fingerprint for UV-B under high nutrient levels was the same as the metabolic fingerprint for UV-B under medium conditions and control radiation under low nutrients.

UV-B had a detrimental effect on *Festuca* under medium nutrient levels ($F = 11.08$; $P=0.003$). Nutrient availability interacted with UV-B with *Galium* ($F=10.07$; $P<0.001$) with UV-B having a significant effect under low nutrient conditions only.

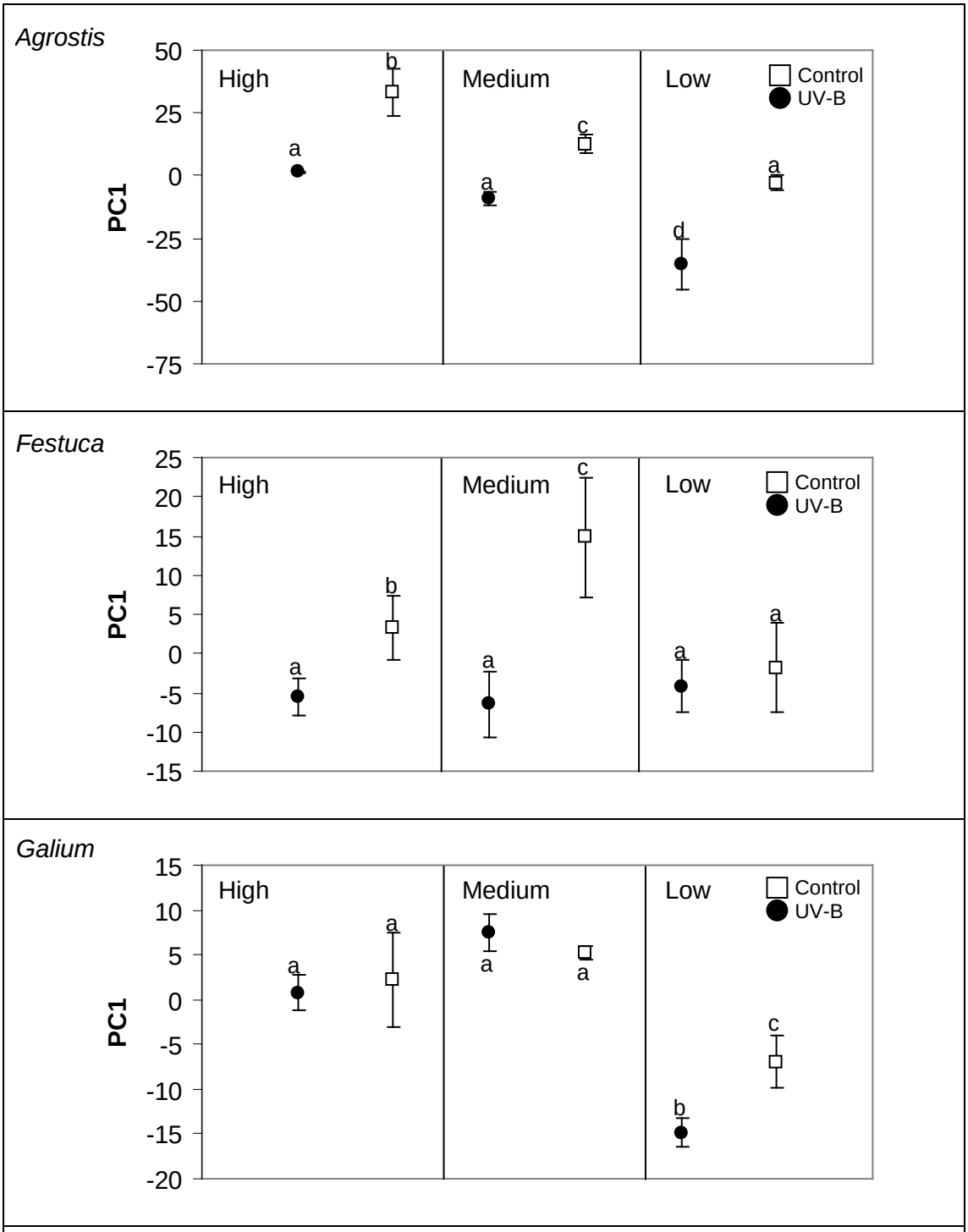


Figure 7.3. Effects of nutrient availability and UV-B on the metabolome of the three species. Letters above standard error bars denote statistical differences.

7.4: Discussion

7.4.1: *The Interaction Between UV-B and nutrient availability*

There was an increase in total community biomass with an increase in soil fertility (Figure 7.2). Barring extreme instances where an excess of fertiliser can be toxic (Britto & Kronzucker, 2002; Bruck & Guo, 2006), this was expected (Fangmeier *et al*., 1994; Krupa, 2003). In terms of nitrogen deposition research, this supports the central hypothesis that pollution is acting as an inadvertent fertiliser by increasing the input of reactive nitrogen (Norby, 1995; Lee 1998; van der Eerden, 1998).

Figure 7.2 also shows that UV-B has a detrimental effect on community biomass in high and medium levels of soil fertility. The damaging effects of UV-B are also well-documented and decreases in community biomass due to ambient levels of UV-B have been observed in a fen ecosystem in Tierra del Fuego, Argentina (Robson *et al*., 2003) and in some species in a sub-arctic heath in Sweden (Johanson *et al*., 1997).

Most importantly, Figure 7.2 shows that the deleterious effects of UV-B are not present under low nutrient conditions. This suggests that the effects of UV-B are dependent on soil nitrogen levels. This effect, whereby plants from nitrogen-limited soils are unaffected by enhanced UV-B, has been observed in other studies involving Mediterranean species (Levizou & Manetas, 2001) and grassland species in the Netherlands (Tosserams *et al*., 2001). Similar results have been obtained from two studies on the Qinghai-Tibetan Plateau with *Acer mono* (Yao & Liu, 2006b) and *Picea asperata* (Yao & Liu, 2007). A study looking at the basis of this phenomenon

by Correia *et al.* (2005) concluded that decreased sensitivity arose from a multi-faceted combination of increased maximal and minimal chlorophyll fluorescence, reduced net photosynthetic rate, increased soluble proteins and higher starch concentration. However, in one study by Deckmyn & Impens (1997) using *Secale cereale* the damaging effects of UV-B were additive and not interactive. Nonetheless, this study provides extra support to the observation that biomass reduction from enhanced UV-B is not significant under nitrogen-limited soils.

These results suggest that habitats limited by nitrogen will appear more tolerant of UV-B. This may explain why UV-B appears to have little effect on sub-arctic communities (Björn *et al*, 1997) as sub-arctic communities are nitrogen-limited (Robinson *et al.*, 1998; Gwynn Jones *et al.*, 1997; Rustad *et al.*, 2001) which may be resulting in the similar mechanism that observed in this experiment. Weih *et al.* (1998) found that nitrogen-use efficiency was increased in an outdoor experiment studying the effects of above-ambient UV-B on *Betula pubescens* subsp. *tortuosa*. It is therefore possible that the increase in nitrogen productivity may be offsetting any potential negative effects under low nutrient conditions. A similar pattern may be occurring in high-arctic sites (Rozema *et al.*, 2006) and Antarctic sites (Huiskes *et al.*, 1999) where the small effects of enhanced UV-B could be attributable to low nutrient conditions. It is therefore important to study the effects of UV-B on non-nitrogen limited communities to test the hypothesis that high-nutrient communities (including intensively fertilised farmland) will be more susceptible to UV-B. Furthermore, it is possible that the effects of UV-B will be more apparent with increased nitrogen deposition.

7.4.2: *Community Structure under High Nutrient Conditions*

By looking at the competition coefficients for high nutrient conditions (Table 7.1) it is apparent that *Agrostis* was the most dominant. This was more pronounced in situations where *Agrostis* was initially sown in high densities. The dominance of grasses in high nutrient conditions was noted by Sebastia (2007) and in other studies focussing on community U4. Stevens *et al*. (2006) found that increased nitrogen resulted in a decline of forbs and an increase in grasses in the UK. Smith *et al*. (1996) also found that fertiliser on sub-montane communities resulted in a shift to *Agrostis*-dominated U4 which suggests that the assemblage as a whole can tolerate high nitrogen conditions. However, in a later study, the same group found that U4 was present in communities under the lowest nitrogen levels of the experiment. Therefore, it is apparent that U4 can grow under a variety of conditions although it is only under high nitrogen conditions that its dominance through a high proportion of *Agrostis* is maintained.

When the results are analysed in the context of nitrogen deposition research, it is clear they support the growing conclusion that high nitrogen conditions favour grasses at the expense of forbs and a decrease in biodiversity (Baddeley *et al*., 1994). Similar studies on neutral-acid / acidic grasslands have shown similar results. The Park Grass experiment at Rothamstead, UK, has been investigating the effects of fertiliser on a grassland since 1856 (Dodd *et al*., 1994). The central conclusion is that high nitrogen conditions lower biodiversity and favour grasses such as *Agrostis* spp.. In Braunton Burrows, UK, a similar pattern was found on dune soils with an increase in *Festuca* (Willis *et al*., 1963) and research from the Somerset Levels has shown a remarkable

decline in forbs and increase in grasses under the higher nitrogen levels (Tallowin & Smith, 1994). However, there have been some exceptions such as in a Norfolk dune system (Boorman & Fuller, 1982) and a notable study involving U4 which showed a lack of effects in the first three years (Morecroft *et al*., 1994). However, on balance the majority of studies suggest that with further long-term studies an increase in grasses with nitrogen deposition can be expected.

Research from calcareous grasslands in this area has been more extensive and has shown similar conclusions. In the Netherlands there has been considerable research (Bobbink & Willems, 1991) with growing concern that nitrogen deposition is responsible for the rapid spread of *Brachypodium pinnatum* (Bobbink *et al*., 1998 b). De Kroon & Bobbink (1997) suggested that up to 75 % of nitrogen locked in an ecosystem could be found in *B. pinnatum*. Part of the success is possibly due to more efficient nitrogen reallocation to roots after senescence (Willems *et al*., 1993). On the other hand, a study in the UK showed that *B. pinnatum* did not pose as great a problem as in the Netherlands under nitrogen deposition treatments (Wilson *et al*., 1995). However, many British calcareous grasslands have seen an increase in grasses with increased nitrogen (Smith *et al.*, 1971; Jeffrey & Pigott, 1973; Morecroft *et al*., 1994). This shows that the dominance of fast-growing grasses under high nutrient-conditions is not restricted to sub-montane habitats.

Research into the effects of nitrogen deposition on heathland communities has also been extensive and has shown substantial international collaboration. The predominant conclusion is that grasses are favoured at the expense of the ericoid species such as *Calluna vulgaris*. Some studies have shown that high nitrogen

encourages grasses such as *Nardus stricta* (Hartley & Amos, 1999), *Deschampsia flexuosa* (Britton *et al*., 2003) and *Molinia caerulea* (Berendse & Aerts, 1994). In the Netherlands it was noted that grasses were mainly dominant when gaps in the *C. vulgaris* canopy appeared (Aerts *et al*., 1990). This consequently increased greater herbivory of *C. vulgaris* (van der Eerden *et al*., 1991). A similar pattern was observed in the British Isles with an increase in grasses always being the ultimate outcome (Barker *et al*., 2004) although on closer inspection it was noticeably where there were gaps in the canopy (Alonso & Hartley, 1998) with a concomitant rise in herbivory (Powers *et al*., 1998). Further work could incorporate herbivory as an additional factor.

The importance of other environmental factors on the effect of nitrogen is also highly important. Britton *et al*. (2003) found that soil type and drought had a greater effect than soil nitrogen on the interaction between *Calluna vulgaris* and *Deschampsia flexuosa*. Frost has also been shown to have an effect on nitrogen sensitivity in *Pinus sylvestris* (Dueck *et al*., 1990) and in the communities of polar semi-deserts in Svalbard (Robinson *et al*., 1998). Acidification of soil from increased nitrogen may also be altering the community structure (Lee *et al*., 1992; Bobbink *et al*., 1998b). Future work could therefore incorporate additional factors such as herbivory and acidification (Wookey *et al*., 1995); Stulen *et al*., 1998). Long term work is equally as important given that the effects of nitrogen deposition are possibly perpetuated in plant litter (Carroll *et al*., 1999).

7.4.3: *Community Structure under Medium and Low Nutrient Conditions*

By studying the competition coefficients (Table 7.1) it appears that *Galium* was most dominant under medium nutrient conditions and more so when it is was initially sown in greater concentrations. Of the three significant coefficients from Table 7.1, all possessed *Galium* under medium nutrient conditions. It would appear that *Galium* benefits from medium nutrient conditions and with a lack of evidence to suggest this is a result of competitive interactions, another hypothesis should be formed. It is possible that the high nutrient levels in the experiment were detrimental to *Galium* (Britto & Kronzucker, 2002; Bruck & Guo, 2006) thereby explaining why it has a lower presence in such communities. Additionally, a study by Sebastia (2007) showed that forbs including *Galium* were more prevalent in low nutrient conditions in a sub-alpine grassland. Further experimentation looking at the effects of soil nutrients on different *Galium* species would test this further.

Table 7.1 also suggests that *Festuca* appears to benefit from low nutrient conditions. Previous studies have shown *Festuca* to predominate on nitrogen-limited soils (Hansson & Göransson, 1993) and be absent on higher nutrient soils (Tuma *et al*., 2005). On the other hand, Michelsen *et al*. (1999) noted that *Festuca* increased with nitrogen addition on a sub-Arctic heath.

7.4.4: *The Role of Competition in Community Structure*

One of the most important conclusions drawn from the results is that competition had little effect on the biomass of the individual species. In the context of the Tilman and

Grime debate (Grace, 1991; Craine 2005), this conclusion superficially supports Grime's hypothesis. One of Grime's central ideas is that competition is not important under stress (Grime, 1977) as witnessed by the lack of significant coefficients. However, this conclusion does not hold up when the providence of the coefficients are studied. Of the nine significant models, three were found under high nutrient soil, four under medium nutrient soil and two under low nutrient soil. Four of these were under UV-B and five were under ambient conditions. There is clearly no pattern as to which conditions a coefficient is significant; on the contrary, the occurrence of competition seems to take place regardless of environmental stress. This naturally would favour Tilman's view although the data does not unequivocally support this hypothesis either. Tilman would predict, unlike Grime, that competition would occur under all environmental conditions (Tilman, 1985) although would also have predicted competition would be more prevalent and not restricted to a limited number of cases. Thus, this experiment suggests competition is not occurring in the early stages of competition which favours neither one hypothesis nor the other and suggests that competition and environmental stress need not always be interactive.

Moreover, the type of competition is also important when studying the coefficients. Seven of the eleven significant coefficients were intraspecific and only three were inter-specific. A similar pattern has been observed elsewhere in this thesis with intra-specific effects being the prevalent type of competition. The fact that monocultures have a lower biomass suggests intraspecific competition is the most intense (Chu *et al*., 2004; Fan *et al*., 2006; Fargione & Tilman 2006). However, it should be noted that intra-specific effects lower the biomass of an individual only and not the community. Therefore, it will only be at its most effective when there is a large

proportion of one species in a mixture anyway. In such cases the species will dominate although the individuals will have a small biomass.

Another important conclusion drawn from the experiment was that the biomass of a community was unaffected by the composition of the species. Thus a community dominated by *Galium* was no more productive than a community dominated by *Agrostis* for any given combination of UV-B and soil fertility as discussed previously. This fits in with data from other studies that have included monocultures in the experimental design. In such studies, the monocultures have a lower biomass than the mixtures that were all similar as in this experiment (Chu *et al*., 2004; Fan *et al*., 2006; Fargione & Tilman, 2006). An advantage of surface-response models is that monocultures are unnecessary and were therefore not employed in this study. However, in not using monocultures the most intense form of competition (intraspecific) was not observed and therefore the most common form of competition was not observed. It is therefore not surprising that there were no community effects in total biomass given the number of experiments where it is only the monocultures that show a significant change in biomass and not the more informative mixtures.

7.4.5: *Metabolic Fingerprinting*

UV-B and nutrient deficiency only affected the metabolome in some of the treatments. For example, the metabolome of *Agrostis* was affected by UV-B under all conditions although *Galium* was only affected under low nutrient conditions (Figure 7.3). Gidman *et al.* (2006) found that the metabolome of *Galium* could be used to predict the nitrogen content of soil although the lack of pattern in *Galium* in this experiment

(see Figure 7.3) suggests this data could not be used for such a purpose despite significant changes being present. Therefore, FT-IR can only detect some changes due to environmental stress and the extent to which it can be used as a tool is limited and will require further analysis.

7.4.6: *Conclusion*

It is apparent that the effects of UV-B and nutrient availability are interactive and that future studies using UV-B should incorporate nutrient availability as a factor. Moreover, the effect of UV-B appears to be limited to total community biomass as there were few effects of UV-B on individual species or competitive ability. Nutrient availability had a more significant effect on community structure with *Agrostis* dominating under high nutrient conditions. Overall, the results suggest that nitrogen deposition will favour fast-growing grasses, such as *Agrostis*, whilst increasing the sensitivity of communities to enhanced UV-B.

7.4.7: *Summary*

1. Response surface designs can effectively analyse more than two species and could be used more widely in multi-species experiments
2. Ambient levels of UV-B detrimentally affect the total community biomass although the effect was negated under low nutrient conditions
3. *Agrostis* dominated under high nutrient conditions with *Galium* and *Festuca* being more prevalent under low nutrient conditions

Chapter Eight: The Effects of Enhanced UV-B on a Sub-Arctic Heath Ecosystem

8.1: Introduction

There has been extensive research into the effects of climate change, such as increased temperature, on arctic plants and communities. The volume of research increased in the early nineties with studies concentrating on individual species such as the heathland *Cassiope tetragona* (Havström *et al*., 1993), mountainous *Dryas octopetala* (Welker *et al*., 1993) and grassland *Calamagrostis lapponica* (Parsons *et al*., 1995). Other studies adopted a more holistic approach by comparing high- and sub-arctic sites (Wookey *et al*., 1993; Havström *et al*., 1993) and the effects on whole heathland communities (Johanson *et al*., 1995a, b).

On the strength of these earlier studies, the mid-nineties saw considerable investment into studying the effects of elevated UV-B and other climatic variables on plant communities. During May 1995, a pan-European research consortium (UVECO) was funded by the EC (grant number: EVSV-CT910032) to further research into the effects of elevated UV-B on natural plant communities. As a result, numerous field-sites were founded or expanded such as the sites at Abisko (Sweden) representing UV-B at 15 % ozone depletion with elevated CO_2 and the 15% ozone depletion representation at Adventalen (Svalbard) (see Gwynn Jones *et al*., 1999a for comprehensive list). Such sites provided the basis from which to further long-term research into the effects of elevated UV-B and factors such as elevated CO_2.

The motivating reason for these studies was essentially due to concerns about global climate change. Evidence of depletion in total column ozone in the Antarctic had been noted in the mid-eighties (Farman *et al*., 1985) and novel satellite imaging presented the formation of regionalised "ozone holes" (Stolarski *et al*., 1986). The damage was linked to anthropogenic release of chlorofluorocarbons (CFCs) such as $CFCl_2$ and $CFCl_3$ which are photodissected at high altitudes releasing chlorine free radicals ($Cl^{\cdot}$) which in turn break down ozone (O_3) (Molina and Rowland, 1974; McElroy *et al*., 1986). This effect is catalysed on ice-particles in polar stratospheric clouds (PSCs) (Molina *et al*., 1987) and exacerbated by sulphuric acid aerosols (Molina *et al*., 1993). With the decrease in the ozone layer it was thought that levels of UV-B, which are largely attenuated by the ozone layer, would increase.

Furthermore, it was noted that damage to the ozone layer was occurring in the Arctic (Kerr, 1988). Reductions of 5 % to 8 % were noted in the Arctic ozone layer (Mount *et al*., 1988; Bruce et al., 1991) and a series of further high-profile studies confirmed the concerns and heightened awareness (Frederick & Snell, 1988; Gleason *et al*., 1993; Müller *et al*., 1997). Concerns were also raised when the exceptionally cold Arctic winter of 1995 to 1996 exacerbated the extent of ozone depletion (Rex *et al*., 1997). It was studies such as these that led to the aforementioned increase in studies into the effects of elevated UV-B in the Arctic.

Another reason why outdoor experiments were set-up was that they represent a more ecologically relevant and realistic scenario than laboratory based experiments. Weih *et al*. (1998) compared the effects of enhanced UV-B on *Betula pubescens* subsp. *tortuosa* under both outdoor and laboratory conditions and found the effects depended

on location of the experiment. This discrepancy was noted by Gwynn Jones *et al*. (1999b) who stated that unrealistic systems of studying UV-B, such as growth cabinets, would result in exaggerated responses. Particular attention was drawn to the exaggerated effects observed in a growth-cabinet experiment by Middleton & Teramura (1994) compared to the more subtle effects noted by Mepstead *et al*. (1996) in a realistic solar-tracking experiment. By studying the effects of enhanced UV-B outdoors, it was hoped that a more realistic picture would emerge into how ozone depletion would affect plants.

After the initial experiments of the early-nineties it was concluded that sub-arctic plants would be more susceptible to enhanced UV-B than other plants. Despite continuous summer daylight, levels of UV-B in the Arctic are far lower than those at lower latitudes. It was therefore hypothesised that Arctic plants would be more sensitive to UV-B as they would have evolved in conditions where UV-B is globally low and would not have been pre-adapted to cope with high UV-B radiation. Additionally, Gwynn Jones *et al*. (1999b) plotted a graph comparing the ratio of daily levels of UV-B to the Leaf Area Index (LAI) from different ecosystems (data from Woodward *et al*., 1995) and found the ratio was higher in Arctic conditions. This was due to the single-species and open canopy structure. Thus the concerns of climate change coupled with the potential susceptibility of Arctic plants, resulted in the considerable scientific investment into enhanced UV-B experimentation.

However, the early indications from the ecosystem experiments suggested that these concerns were largely unfounded. There was a clear lack of significant growth response from the UV-B and CO_2 interaction experiment although changes in berry

production herbivory were notable (Gwynn Jones *et al*., 1997). Moreover, after two more years (Gwynn Jones *et al*., 1999a) the status quo was largely maintained. A notable exception was found by Johanson *et al*. (1995a) who observed UV-B mediated differences to dwarf shrub growth. Despite this, in a comparison of different field sites after the first few years, Björn *et al*. (1997) concluded that there was a lack of evidence that the hypothesised susceptibility would occur.

The consensus among researchers following the initial lack of response was that changes could only be expected after a prolonged period of time. Gwynn Jones *et al*. (1997) suggested that changes to community biomass due to UV-B may even take one century to result in a community alteration which necessitates continual monitoring. Björn *et al*. (1997) similarly concluded that it was only with long-term exposure that the realistic effects of enhanced UV-B would be observed. Thus after a decade of exposure it is still necessary to note any changes and continual monitoring is needed.

A further advantage of maintaining the Arctic sites is that they provide a basis from which to test any relevant novel hypotheses. Whilst the original emphasis was on aboveground communities the focus shifted to belowground processes and the continual long-term maintenance of the sites allowed such belowground work to be carried out. Johnson *et al*. (2002) found UV-B mediated changes in belowground organisms that were far more striking than the relative lack of response aboveground. A potential hypothesis is that UV-B up-regulates the production of plant flavonoid production as a protection against radiation (De La Rosa *et al*. 2001; 2003). Such compounds are known to alter soil micro-organism structure when exuded by roots (Siqueira *et al*., 1991; Chabot *et al*., 1992). It can be hypothesised that should UV-B

alter the quantity and quality of plant flavonoids, the belowground organisms may be affected. The field sites provide the opportunity to harvest leachates and test such hypotheses.

Therefore, if the full potential of the early-nineties scientific investment into the Arctic is to be realised, then work must continue to monitor the effects. The following experiment is a continuation of the sub-Arctic research and will provided additional data into the ongoing attempts to understand the effects of enhanced UV-B. This chapter aims to address three key issues that have been raised by research so far. Firstly, it is important to find out whether the initial concerns raised by stratospheric ozone depletion are exacerbated by the progress of time. Secondly, it is crucial to understand whether the role of belowground processes is more important than aboveground processes. Thirdly, and commensurate with the central theme of the thesis, the potential use of metabolomics in detecting UV-B changes should be assessed.

8.1.1: *Aims*

1. Test the hypothesis that long-term (15 years) exposure to enhanced UV-B alters dwarf shrub biomass in a sub-arctic heath
2. Test the hypothesis that UV-B alters the metabolic profile of soil leachates
3. Test the hypothesis that any effects of enhanced UV-B can be detected by metabolic fingerprinting of sub-arctic heath species

8.2: Materials and Methods

8.2.1: *Experimental Set-Up*

The effects of enhanced UV-B on a sub-Arctic heath ecosystem were tested at a field irradiation system established in 1991 at Abisko, Swedish Lapland (68.35°N, 18.82°E, 360 m a.s.l.). The community consists of a ground layer containing mosses and lichens, an ericaceous shrub layer containing of two evergreen species (*Empetrum hermaphroditum* and *Vaccinium vitis-idaea*) and two deciduous species (*V. myrtillus* and *V. uliginosum*), and a tree layer containing *Betula pubescens* subsp. *tortuosa*.

The site consists of eight metal frames (2.5 x 1.3 x 1.5 m) and four similarly sized plots (2.5 x 1.3 m) which had no metal frames and acted as outside controls. Each of the metal frames contained six fluorescent lamps (Q-Panel, UVB-313, Cleveland, OH, USA) with the central 70 cm of the middle two lamps covered with aluminium foil to ensure even distribution of light. All lamps were pre-burned for 100 hours to ensure stability of output.

The eight metal frames were divided into four controls and four acting as elevated UV-B treatments (representing 15% ozone depletion). The four control frames possessed window glass to attenuate the UV-B although ensure that the properties of fluorescent tubes (e.g. UV-A, PAR, flicker-rate) were still present in the control treatment. The four enhanced UV-B frames were filtered by 0.13 mm cellulose diacetate (CA) (Curtails, Derby, UK) to remove any ecologically irrelevant UV-C (<

280 nm). The CA was attached to a UV-transmitting Plexiglas covering (Röhm 2455, Röhm GmbH, Darmstadt, Germany).

The treatment begins each year before all the snow has thawed (late April to mid-May depending on severity of winter) and continues until mid-September. The 15 % ozone depletion scenario was calculated by giving a daily dose of UV-B_{BE} according to Björn & Murphy's (1985) solar UV-B model. The dose was administered at noon and was controlled by an automatic time-switch that turns three-lights on at a time in a step-wise pattern. Every second week the exposure time is altered.

8.2.2: *Leachate Set-Up*

Soil leachates were collected from each plot. Two Rhizon soil moisture samplers (Van Walt Ltd., Haslemere, UK) were inserted into the soil in opposite corners of the plot. Each Rhizon consisted of a porous cup, a connecting tube and a syringe to keep the system under negative pressure. The diameter of the tube was 2.5 mm which ensured minimal disturbance and was constructed from inert material to ensure no ion exchange or alterations in pH occurred. One hour prior to the samples being taken, the soil was wetted with 500 ml of distilled water in order to ensure a liquid sample was taken (the soil would be too dry otherwise). Trial experiments showed that 500 ml was the minimum amount to be added which would ensure a sample could be taken after one hour. After the wetting treatment, the Rhizon syringes were then depressed and held in place to harvest the soil leachates. The leachates were then stored at -80°C for three months until analysis for Total Organic Carbon.

8.2.3: *Growth Measurement Analysis*

Destructive measurements were taken from each of the twelve plots (four enhanced UV-B, four framed controls and four outside controls). For each plot, ten stems from each of the four species were cut at the start of the previous year's growth. For *E. hermaphroditum*, *V. myrtillus* and *V. vitis-idaea* the stem from 2005 with the current year's (2006) growth (formed as a number of stems from the top of the previous year) were taken. As *V. uliginosum* had not shown signs of growth in 2006, the stem from 2004 with the following year's growth was taken instead.

The oldest stem (2004 for *V. uliginosum* and 2005 for the rest) was measured in mm and the number of stems sprouting from the top were counted. The average length (mm) and biomass (mg) of these stems was recorded. The area (cm^2) and thickness ($mm*10^{-3}$) of the leaves from the *Vaccinium* species was recorded. Leaves from *E. hermaphroditum* were not measured given their small and barrel-needle shape. Each parameter was analysed using a one-way ANOVA with three levels (control frames, enhanced UV-B, outside control; n=4) for each of the four-species.

8.2.4: *Leachate Analysis*

The leachates were then analysed for Total Organic Carbon (TOC). It was measured in a stand-alone TOC-V Analyzer (Shimadzu, Columbia, MD, USA) which initially measured Total Carbon (TC) before subtracting the proportion that was inorganic (IC) to give TOC. The system measures both TC and IC by measuring CO_2 absorbance using an infrared source and quantifying amounts according to a pre-programmed

calibration curve. The TOC (measured in mg/l) from the plots was then compared using one-way ANOVA.

8.2.5: *Metabolic fingerprinting*

Four of the current year's stems were taken from each species and treatment for metabolic fingerprinting. The total complement of leaves was ground in a mill and 50 mg of the powder was mixed with 50 µl of distilled H_2O for analysis by FT-IR. Analysis by FT-IR and chemometric analysis was carried out according to the protocol in Chapter Two.

8.3: Results

8.3.1: *Biomass Measurements*

Of the four species, the evergreen *V. vitis-idaea* (Figure 8.1) showed the greatest overall growth for all treatments with the similarly evergreen *E. hermaphroditum* showing the least growth (Figure 8.2). Leaf biomass for *V. vitis-idaea* was 111.6 % higher than *E. hermaphroditum* with the deciduous *V. myrtillus* and *V. uliginosum* having a similar and intermediate leaf biomass between the two evergreens. *V. vitis-idaea* also had the greatest stem length for the two years (Figure 8.3) although *V. myrtillus* had the greatest leaf area and number of stems (Figure 8.1).

The results from Figures 8.2 & 8.3 also show that there were no adverse effects of enhanced UV-B on the two evergreen species (*E. hermaphroditum* and *V. vitis-idaea*).

There was a notable decrease in 2005 stem length of 13.6% due to enhanced UV-B in *V. vitis-idaea*, although the large standard error of the means for the control frame (5.2 compared to 1.5 for the enhanced UV-B frame) meant that no statistical differences were present. No other such patterns were evident and it appears that evergreens are largely unaffected by enhanced UV-B.

The deciduous *V. uliginosum* was similarly unaffected by enhanced UV-B (Figure 8.4) although *V. myrtillus* was greatly affected (Figure 8.1). Whilst UV-B had no significant effect on the most recent stem length (2006), the previous year showed a significant decrease of 67.2 % (figure 8.1). The number of stems branching from the previous year's growth was reduced by 47.2 % (Figure 8.1) with mean stem biomass 189.4 % lower. This suggests that not only does UV-B damage the potential for new growth from old stems but severely stunts the new growth also. Leaf thickness was unaffected by enhanced UV-B although leaf area was decreased by 117.6 % (Figure 8.1) and leaf biomass was decreased by 132.9 % (Figure 8.1). Therefore, enhanced UV-B affected shoots of the deciduous *V. myrtillus* but not *V. uliginosum* suggesting a species-specific response to UV-B stress.

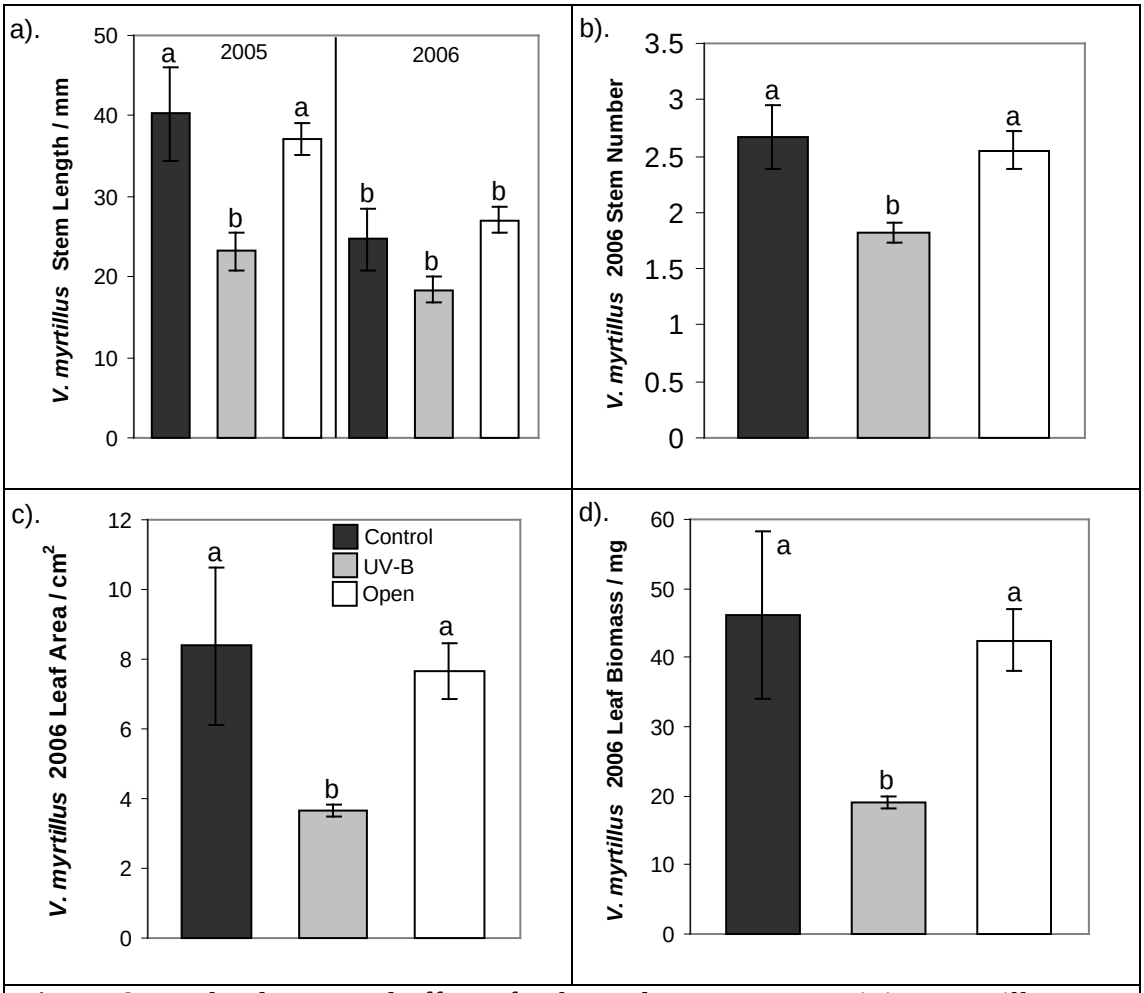


Figure 8.1. The detrimental effect of enhanced UV-B on *Vaccinium myrtillus* stem length in 2005 and 2006 (a) and stem number (b), leaf area (c) and leaf biomass for 2006 (d). Control frames are shown in dark grey, UV-B frames in light grey and open plots in white. Different letters denote statistical differences ($P<0.001$)

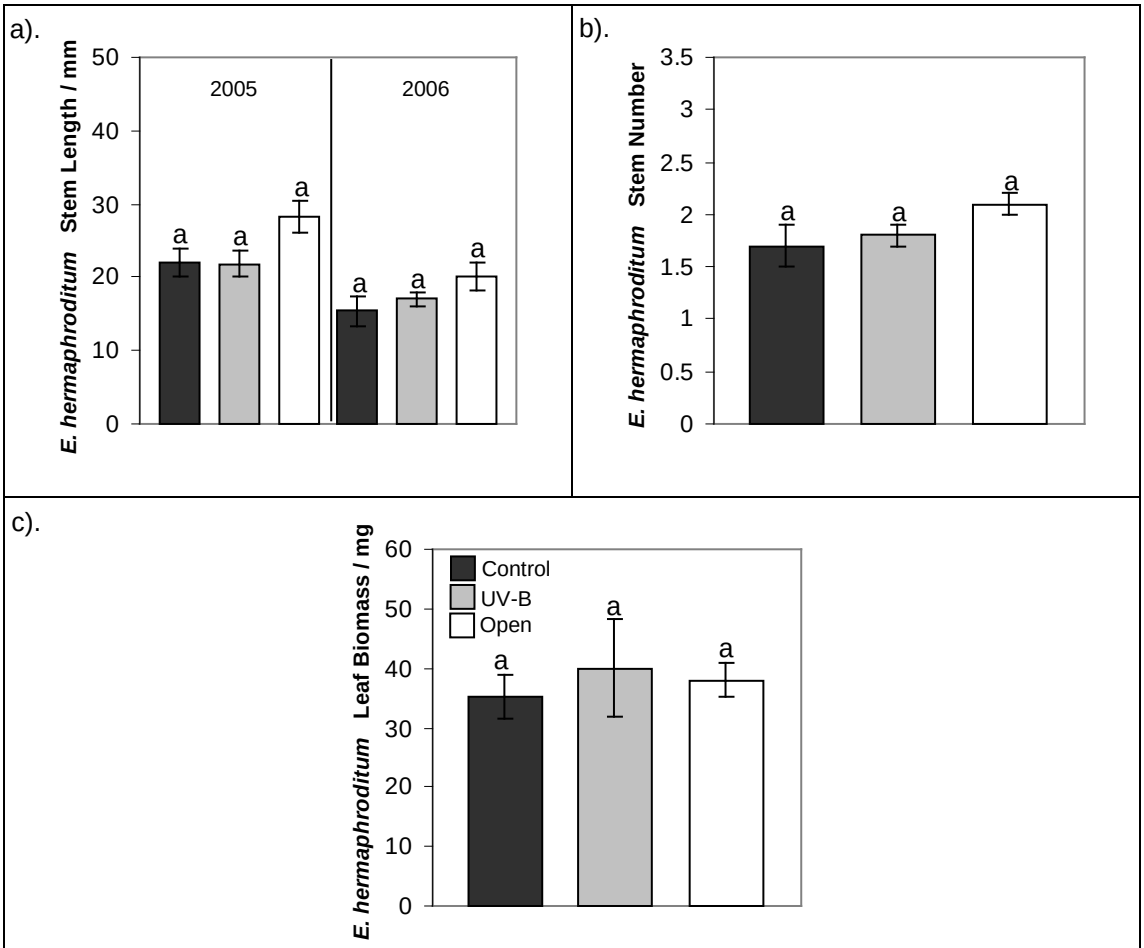


Figure 8.2. The effect of enhanced UV-B on *Empetrum hermaphroditum* stem length in 2005 and 2006 (a) and stem number (b) and leaf biomass for 2006 (c). Control frames are shown in dark grey, UV-B frames in light grey and open plots in white. Different letters denote statistical differences ($P<0.001$). Due to the small needle-shaped leaves of this species, area was not taken.

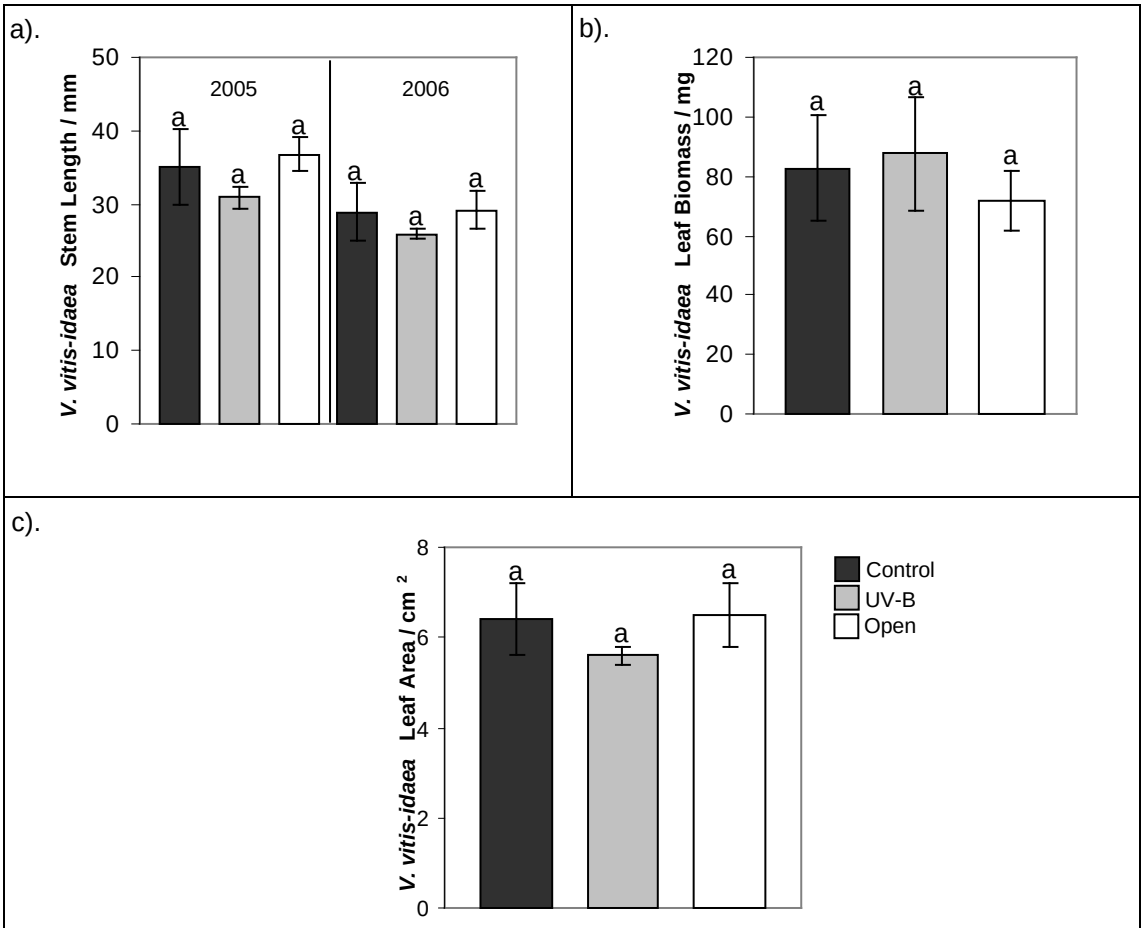


Figure 8.3. The effect of enhanced UV-B on *Vaccinium vitis-idaea* stem length in 2005 and 2006 (a), leaf biomass (b) and leaf area (c). As there was only one stem, no data is presented for this factor.

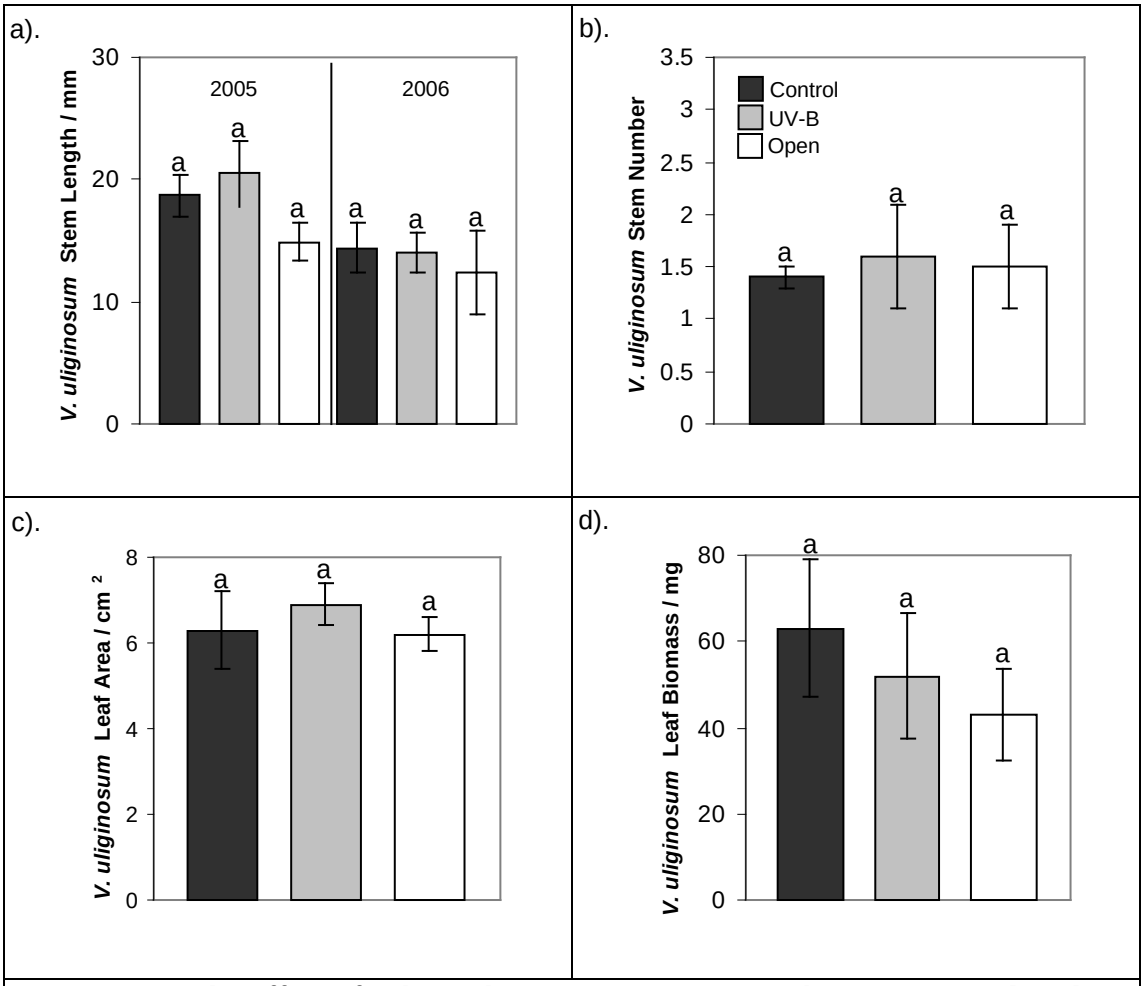


Figure 8.4. The effect of enhanced UV-B on *Vaccinium uliginosum* stem length in 2005 and 2006 (a) and stem number (b), leaf area (c) and leaf biomass for 2006 (d). Control frames are shown in dark grey, UV-B frames in light grey and open plots in white. Different letters denote statistical differences ($P<0.001$)

8.3.2: *Soil Leachate*

Analysis of the soil leachates showed that there was a sufficient level of total organic carbon (TOC) present in the soil for accurate analysis to be carried out (with a range of approximately 80 to 120 mg/l). Nonetheless, the level of TOC did not vary significantly due to radiation treatment (Figure 8.5a). Figure 8.3b shows the proportion of total carbon (TC) for the three treatments (enhanced UV-B, control frame, outside control) that is comprised of organic (TOC) and inorganic carbon (IC). No significant differences were present in the ratios of TOC to IC.

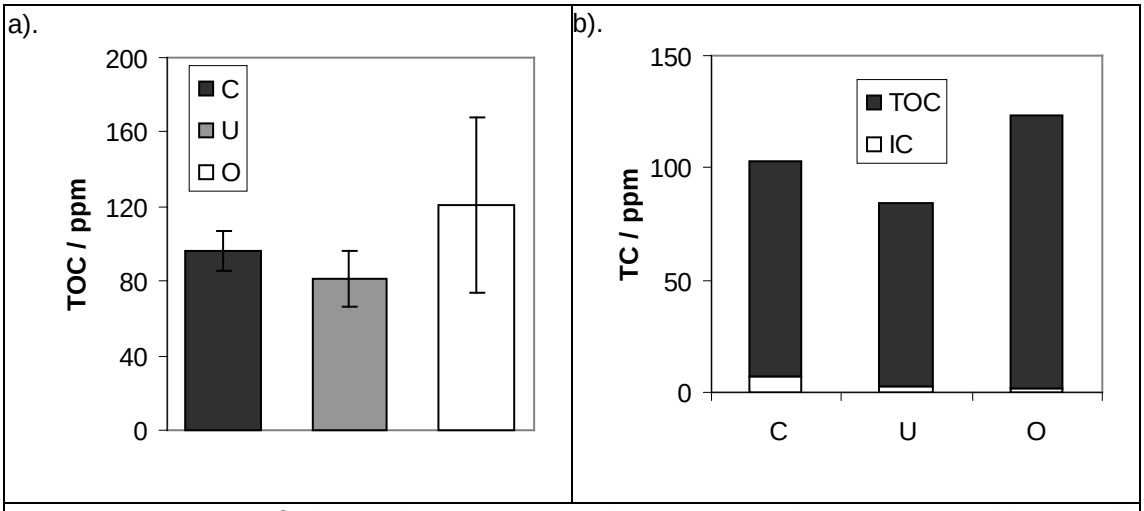


Figure 8.5. Pair of charts showing a). Total Organic Carbon (TOC) and b). Total carbon (TC) for control frames (C), enhanced UV-B frames (U) and designated areas outside frames (O). For each treatment, Total Carbon (TC) is divided into total Organic Carbon (TOC) and Inorganic Carbon (IC). No significant differences.

8.3.3: *Metabolic Fingerprinting of Dwarf Shrubs*

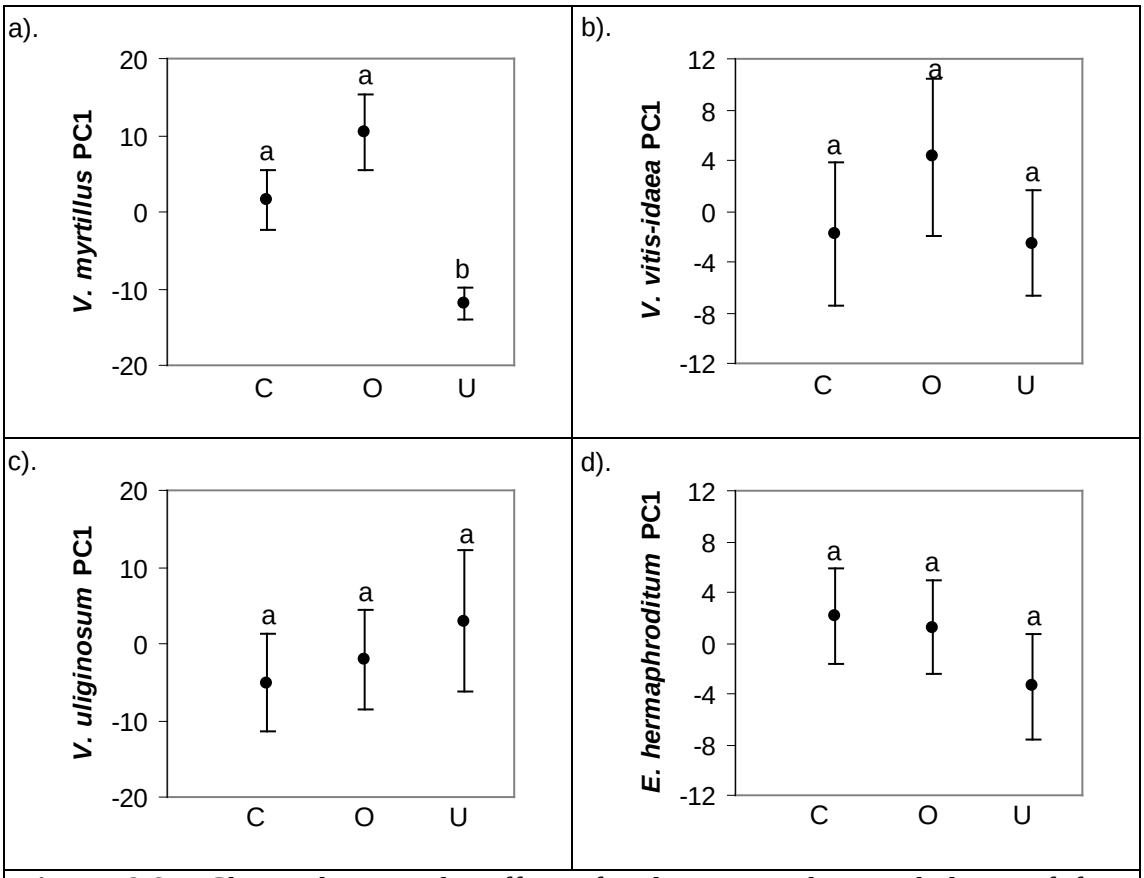


Figure 8.6. Charts showing the effect of radiation on the metabolome of four heathland species (a. *Vaccinium myrtilus*; b. *V. vitis-idaea*; c. *V. uliginosum*; d. *Empetrum hermaphroditum*). Values of PC1 are presented according to conditions of: C – control; O – outside plot and U – enhanced UV-B. Letters above SE bars denote similarities and differences; the only difference in this case being between enhanced UV-B and control conditions in *V. myrtillus* ($P<0.001$).

The metabolic fingerprints of *V. vitis-idaea*, *V. uliginosum* and *E. hermaphroditum* were unaffected by enhanced UV-B. This corresponds to the lack of growth parameters affected by UV-B (Figures 8.2, 8.3 & 8.4). The metabolic fingerprint of *V. myrtillus* was affected by UV-B with the samples from UV-B frames being statistically different ($P<0.001$) from the controls (Figure 8.6a). However, if the spectral replicates are averaged to give one representative spectrum per frame then the

significant effect of UV-B on *V. myrtillus* is lost and a significant UV-B mediated effect is noted with *V. vitis-idaea* instead. In this experiment, the values for the individual spectra were used (thus suggesting *V. mytrtillus* was affected) as doing subsequent analysis on averaged figures introduces more error and uncertainty. Nonetheless, this exemplifies that the chemometric analysis is of paramount importance to the way the data is interpreted.

8.4: Discussion

8.4.1: *Comparison of Previous Studies from the Same Experimental Plots*

Given that a similar experiment had been carried out on the same plots ten years previously (Johanson *et al*., 1995 a, b), it is important to compare the two sets of data to assess whether the effects of UV-B are cumulative. However, it is equally important to compare the community structure of the control plots to see whether the dwarf-shrub heath naturally varies with time. In 1995 (Johanson *et al*., a, b) it was concluded that the deciduous species were dominant over the evergreen species. Leaf biomass of *V. myrtillus* was 35 % greater than *V. uliginosum* and 23 % higher than *V. vitis-idaea*. In this experiment, it was the evergreen *V. vitis-idaea* that was dominant showing over an one-hundred percent increase in leaf biomass over the other species (Figure 8.3). However, the evergreen *E. hermaphroditum* possessed the lowest leaf biomass of all species (Figure 8.2). This suggests that deciduous species are no longer the dominant species as the evergreen *V. vitis-idaea* possessed the greatest biomass. It must be said that *V. myrtillus* showed the greatest leaf area and branching number (Figure 8.1) although it is clearly *V. vitis-idaea* that is most abundant in terms

of biomass. This comparison draws two conclusions: (1) that deciduous species are no longer dominant in the sub-Arctic heath and (2) that community structure shifts over time.

In comparing the effects of enhanced UV-B between the two experiments it becomes apparent that the results from this study share more in common with Johanson *et al*. (1995, a, b). *V. myrtillus* was the only plant to be affected by UV-B in both studies. Johanson *et al.* (1995, a, b) noted a 15 % decrease in leaf biomass whilst this study noted an over two-hundred percent reduction in stem biomass, an over one-hundred percent reduction in leaf biomass and an over fifty percent reduction in branch numbers (Figure 8.1). This decline is very much greater and suggests a cumulative negative response over time.

As *V. myrtillus* was the only species affected, Johanson *et al*. (1995a) predicted a decline in the dominant deciduous species at the expense of the evergreens. These data suggest a further decline in the deciduous *V. myrtillus* at the expense of the currently dominant evergreen *V. vitis-idaea*. However, it is important to not put too much emphasis on the functional classification of species being deciduous or evergreen as favoured by Johanson *et al*. (1995, a, b) given that both the deciduous *V. uliginosum* and evergreen *E. hermaphroditum* were unaffected by enhanced UV-B. In the context of the control plots, it is also possible that a natural increase in ambient UV-B due to climate change has resulted in the increase in *V. vitis-idaea* as predicted by Johanson *et al.* (1995, a, b). However, as there has been no appropriate control to attenuate ambient UV-B it is unknown whether this shift is natural or UV-B mediated.

A study by Gwynn Jones *et al*. (1997) conducted at another site at Abisko showed no negative effects of enhanced UV-B on the growth of any of the dwarf shrubs. They suggested that the negative effects observed by Johanson *et al*. (1995, a, b) were due to lesser snow-cover at that site and the fact that exposure times began before all the snow had thawed. This would increase the amount of UV-B the plants received and explain why *V. myrtillus* was more affected in this study and with Johanson *et al.* (1995, a, b).

However, Gwynn Jones *et al*. (1997) did notice that enhanced UV-B increased berry production in *V. myrtillus* and herbivory of its leaves. The net effect of the positive increase in berry production (assuming the berries were fertile) and negative effect of herbivory may be affected by location. At the sheltered site of Gwynn Jones *et al.* (1997), the lack of any negative effects of UV-B and positive effect of berry production may mitigate the negative effect of increased herbivory. On the more xeric site of Johanson *et al*. (1995, a, b) and this study, the negative effect of UV-B and potential increase in herbivory could outweigh the potential increase in berries and tip the balance in favour of more resistant species.

Other factors that could affect the extent of damage by enhanced UV-B have also been studied at Abisko. The aforementioned study by Gwynn Jones *et al*. (1997) studied interactions with enhanced CO_2 although no significant effects were observed. The effects of enhanced UV-B on regeneration were also tested at the same site as this study (Phoenix *et al*., 2000). A patch of vegetation was completely denuded and it was observed that *V. myrtillus* showed enhanced regeneration under elevated UV-B. Thus should the heath ever be denuded (e.g. increased herbivory) then *V. myrtillus*

may perversely benefit from the enhanced UV-B in the initial regenerative stages although be disadvantaged when the heath is more established. A further study by Phoenix *et al*. (2001) studied the effects of increased precipitation although, like the enhanced CO_2 plots, no significant interaction was present. However, Phoenix *et al*. (2001) did note a decrease in biomass of *V. myrtillus* in some years which corroborates results from this study. Thus whilst enhanced UV-B may act independently of some environmental factors, the results from the regeneration experiment suggest the effects on enhanced UV-B could depend on other factors.

The effects of enhanced UV-B on leaf thickness have also been extensively studied at Abisko. Johanson *et al.* (1995, a, b) noted that leaf thickness decreased in deciduous species although increased in the evergreen *V. vitis-idaea*. Conversely, Phoenix *et al*. (2001) noted that the deciduous *V. myrtillus* showed increased leaf thickness in some years. The lack of changes to leaf thickness noted in this study was similar to the lack of effects observed by Gwynn Jones *et al*. (1997). The role of altered leaf thickness may be of importance to the effect of light-mediated alterations to herbivory (Roberts & Paul, 2006) and is worth directly testing. However, there is still no current consensus on how UV-B affects leaf thickness given that different species are affected at different times in different ways. It is only with more research that the mechanism and effects of UV-B mediated changes to leaf thickness will be discovered.

8.4.2: *Effect of UV-B on Belowground Parameters*

The role of belowground parameters was also tested in this experiment by the sampling of soil leachates. Whilst organic carbon was found in sufficient quantities

for analysis to take place, there was no difference due to enhanced UV-B. However, previous studies at Abisko have shown that belowground parameters are particularly important. An early study by Gehrke *et al*. (1995) began to show that shifts in soil microbial respiration were occurring due to enhanced UV-B. Johnson *et al*. (2002) found great alterations in soil microbial biomass. More recently, Rinnan et al. (2006) found enhanced UV-B decreased monocarboxylic acid in exudates in *Narthecium ossifragum* although increased it in *Eriophorum angustifolium* with 15 % ozone depletion over eight weeks. Moreover, data from Tierra del Fuego suggest that soil micro-organisms are more affected than the vegetation (Searles *et al*., 2001; Zaller *et al*., 2002). Given the limited time frame of the exudate collection in this experiment it is not surprising that no patterns were observed. A more extensive network of leachate-harvesting Rhizons and regular collection of leachates are required followed by more rigorous chemical analysis in order to gain a more comprehensive understanding of belowground processes.

Such rigorous chemical analysis could be achieved by metabolomics. However, in this experiment no chemical detection was achieved when using the highly sensitive ESI-MS (data not shown). This suggests that until an alternative method is found, for example NMR, then TOC analysis is perhaps the most informative technique that is widely used.

With regards to metabolic fingerprinting of plant leaf material, the only species to be altered metabolomically (*V. myrtillus*) was also the only species affected morphologically (Figure 8.1). Presumably, the damage to the leaves was chemically detectable in the metabolome. Likewise, the lack of morphological change in the

other species corresponds to a lack of change in the metabolome. This suggests that the plants had a constitutive defence against UV-B. However, as stated in the results, the outcome of the results depended on statistical analysis. Averaging the spectra for each of the four replicates from each plot resulted in a lack of metabolomic effect in *V. myrtillus* but a change in *V. vitis-idaea*. This underlines the importance of a standardised statistical protocol throughout the metabolomic community in order to avoid bias. Such issues and comparison of the metabolomic results in the context of the other studies are presented in the final discussion.

A further effect of enhanced UV-B on dwarf-shrubs could be via altered decomposition of leaves. Gehrke *et al*. (1995) found altered chemical composition of both deciduous species. There was an increase in tannins and decrease in α-cellulose which corresponded to decreased microbial respiration. The group concluded that decreased leaf decomposition would slow nutrient-turnover. As this study has shown *V. myrtillus* to have changed more dramatically compared to earlier studies it would be interesting to see whether the effects of decomposition noted by Gehrke *et al*. (1995) have become more exacerbated. Further experiments could concentrate on how decreased nutrient turnover affects community structure in the field and whether this would in turn be affected by enhanced UV-B. To date this has not been directly tested and would fill an important gap in the literature.

8.4.3: *Comparison of Similar Studies from Different Ecosystems: Towards a Synthesis of The Global Effects of UV-B on Natural Ecosystems*

Given the global nature of the research effort it is informative to analyse the results of this experiment in the context of related sites from around the world. Similar Arctic studies have been carried out at higher latitudes in Adventdalen (Svalbard) (Rozema *et al*., 2006) and Greenland (Albert *et al*., 2005). The conclusion after seven years of study in Svalbard was that enhanced UV-B did not affect the growth of vegetation which concurs with results from Abisko (Rozema *et al*., 2006). However, the results from Solheim *et al*. (2002) did note a change in nitrogen-fixing potential of cryptograms in Adventdalen that was not observed in Abisko suggesting the effects in the High Arctic may be more pronounced over time. Two studies based in Greenland showed more significant changes than at Adventdalen. Photosynthetic efficiency of *Salix arctica* and *V. uliginosum* were significantly decreased with UV-B (Bredahl *et al*., 2004; Albert *et al*., 2005). It is notable that *V. uliginosum* was affected by UV-B given that, along with *E. hermaphroditum*, it has shown little response in Abisko. Nonetheless, the results from the High Arctic sites have drawn similar conclusions to Abisko: that small changes are present although the net effect appears negligible.

Results from the climatically similar although geographically isolated Antarctic suggest that the effects of enhanced UV-B may be greater than those in the Arctic. Xiong *et al*. (2002) noted a reduction of 29 % in biomass of *Colobanthus quitensis* which was also noted to be susceptible to enhanced UV-B in a four-year study by Day *et al*. (2001). Dunn *et al*. (2002) concluded that native Antarctic species may be more at risk than cosmopolitan species. The native moss *Schistidium antarctici* was

significantly affected by UV-B whilst the cosmopolitan mosses *Bryum pseudotriquetrum* and *Ceratodum purpureus* were unaffected. The group thus predicted that enhanced UV-B will favour cosmopolitan species at the expense of endemics. A general review of the effects of UV-B on the Antarctic by Huiskes *et al*. (1999) came to a similar conclusion to researchers at Abisko in that changes were negligible although with an increasing number of studies such as those by Day *et al*. (2001), Dunn *et al*. (2002) and Xiong *et al.* (2002) it is possible that the greatest effects of enhanced UV-B will be observed in the Southern Hemisphere.

Whilst Abisko possesses the longest-running enhanced UV-B site in current operation, a more recently established facility in Tierra del Fuego, Argentina, has produced extensive results. The vegetation of South Argentina is dominated by *Nothofagus* spp. forests and *Sphagnum* spp.-dominated bogs and hence the majority of work is carried out on these species (much as the *Vaccinium* species are studied in Abisko). A remarkably similar pattern to Abisko has emerged. The initial findings found that there were no effects of natural UV-B on primary productivity (Ballaré *et al*., 1999; Searles *et al*., 1999). However, the early studies, much like Abisko, noted alterations to herbivory. Rousseaux *et al*. (1998; 2001) noted that attenuating UV-B increased palatability of *Gunnera magellanica* by up to 75 %. Moreover, like Abisko, longer-term studies have bolstered the early indications that no vast changes were occurring (Searles *et al*., 2002; Robson *et al*., 2003). However, and once again similar to Abisko, it is suggested that soil microbial communities show a greater response to UV-B than above-ground communities (Searles *et al*., 2001). Zaller *et al*. (2002) noted that attenuating natural UV-B decreased symbiotic mycorrhizae formation. Therefore both Abisko and Tierra del Fuego have come to the same general

conclusions: that plants are largely immune to enhanced UV-B; that herbivory is altered and that belowground processes may be the most at risk.

Therefore, commentators on the effects of enhanced UV-B on a global scale have come to the same conclusion that enhanced or natural UV-B does not significantly affect primary productivity (Sullivan, 1997; Rozema, 1999; Aphalo, 2003). The general consensus is that small, species-specific morphological changes may elicit alterations on a longer time-scale. On this basis continued study is needed. The results also shed light on the revisionist view that studies into the effects of enhanced UV-B bear ecological relevance to areas where UV-B is naturally high (Paul, 2001; Paul & Gwynn Jones, 2003 & Aphalo, 2003). Given that only small changes with enhanced UV-B have been observed it is still unknown how great the effects of natural UV-B is. The ecosystems discussed so far have been quite diverse and thus it would appear plants throughout the world share a similar high tolerance to UV-B. It is with studies that attenuate natural UV-B, such as in Tierra del Fuego, that the effects of ambient UV-B will be discovered.

8.4.4: *Conclusion*

In conclusion, the results from this experiment suggest that enhanced UV-B does not have a marked effect on the functioning of a sub-Arctic heath ecosystem. This is in accordance with other studies based at Abisko. However, the relatively large species-specific effect on *V. myrtillus* suggests that the effects of enhanced UV-B could be accumulative and warrants the continued impact of long-term effects to be assessed. Moreover, in the context of global research, the results corroborate the recurring

pattern that effects are limited to small morphological changes in species-specific circumstances. The leachate studies, whilst of limited relevance in this experiment, show that extensive Rhizon-based studies in the sub-Arctic would be desirable, especially given the growing body of evidence that UV-B has a greater effect below-ground than above-ground. Lastly, it is noted that metabolomic fingerprinting may mirror above-ground morphological changes although continued statistical reassessment is required.

8.4.5: *Summary*

1. UV-B detrimentally affected *Vaccinium myrtillus* which was also the only species to show a response on the same experimental plot ten years previously
2. *Vaccinium myrtillus* was also the only species to show a detectable response to UV-B using FT-IR which suggests that this can be used a potential tool in screening for alterations due to enhanced UV-B
3. Whilst UV-B has been hypothesised to alter belowground parameters, TOC and FT-IR analysis showed no differences due to enhanced UV-B

Chapter Nine: Discussion

9.1: General Discussion

This project aimed to test two hypotheses the first of which was that UV-B would alter the competitive balance between two competing species by having a different effect on each group. The second was that metabolic fingerprinting could be used to detect the extent of abiotic stress and competition. By testing these hypotheses it became apparent that a mechanism other than nitrogen-fixation was causing the facilitative effect between *L. perenne* and *L. corniculatus*. The results suggested that alterations to intraspecific competition were mainly responsible. This discussion analyses the two initial hypotheses separately and then discusses the possible mechanisms for overyielding with suggestions for future work.

9.1.1: *The Effects of UV-B on Plant Competition*

There is evidence to suggest that enhanced UV-B affects the competitive balance between two species. This was most clearly seen in the two outdoor enhanced UV-B experiments. In the sub-arctic (Chapter Eight), *V. myrtillus* showed significant reductions to biomass which may result in a future increase in other cohabiting species (Johanson *et al.*, 1995a, b). In the artificial plant community experiment (Chapter Five), *L. corniculatus* was noticeably sensitive to UV-B in both monocultures and mixtures with significant reductions to biomass (Figure 5.1). *L. perenne*, on the other hand, did not show any reduction to biomass (Figure 5.1).

It has been noted elsewhere that UV-B is more damaging to broader-leafed species than thin-leaved species predominantly because there is a greater area for UV-B to damage (Sullivan & Teramura, 1992; Liakoura *et al.*, 1997; Nagel *et al.*, 1998; Keiller & Holmes, 2001). Furthermore, Barnes *et al.* (1990a, b; 1995), who have contributed the greater part of the work on UV-B and competition to date, also predicted that competition would alter depending on the effects of leaf morphology. However, the research in this thesis differs from Barnes *et al.* (1995) in that the main reason for the increase in *L. perenne* was because it was more tolerant of UV-B stress as opposed to beneficial alterations to leaf morphology (Chapter Five). These differences could be attributable to differences in the dose of UV-B. In this study, UV-B was being used as a stress and the dose was relatively high (Table 2.1) (12.2 $KJ/m^2/day^-1$) compared to the comparatively low dose of Barnes *et* al. (1996) (between 4.54 and 5.66 $KJ/m^2/day^{-1}$; data from Chapter Two). In either case, it is clear that UV-B is likely to alter plant competition with communities shifting to those species more tolerant of UV-B.

The two experiments that used ambient UV-B in growth cabinets, namely the artificial U4 sub-montane experiment (Chapter Seven) and mutant experiment (Chapter Six), showed little effects of UV-B. In the sub-montane experiment, UV-B only differed from control conditions in that the intraspecific coefficient in low nutrient conditions was significant (Table 7.1) which suggests that the simulated ambient levels of UV-B had little effect. UV-B also had a negligible effect in the mutant experiment where the non-nodulating mutants had a greater biomass than the non-UV-B control. This suggests that UV-B does not have a substantial effect on plant interaction at ambient levels. This agrees with Björn *et al.* (1997) who

concluded that the effects of UV-B or low supplemental irradiances are very small on plant communities and that most plants are able to tolerate it. It is only when UV-B is substantially above ambient, as in the outdoor experiments, that UV-B will alter plant dynamics.

The theories of Tilman (1985; 1987; 1990) and Grime (1974; 1977; 1989) were directly tested in Chapter Five, and whilst the evidence superficially supported Grime's theory, it is apparent that the evidence does not entirely support one theory or the other. For example, the outdoor UV-B experiment was highly damaging to *L. corniculatus* and it reduced both the intra- and interspecific competition coefficients (Table 5.2). This supports Grime's view that competition is only important in the absence of stress. However, *L. perenne* was unaffected in the same communities and continued to show significant competition coefficients (Table 5.2) which supports Tilman's view that competition occurs irrespective of stress. It can be argued that *L. perenne* was not experiencing stress as there was no decrease in biomass (Table 5.2) which would satisfy Grime's theory. However, the theory should therefore accommodate the view that stress selection is on the species-level as opposed to the community-level. Another interesting phenomenon was that in the sub-montane experiment (Chapter Seven), the competition coefficients were not always significant (Table 7.1) which suggests that initial seeding was the main determinant of the community structure as opposed to alterations between stress tolerance and competition. The fact that stresses can affect the component species of a community in different ways may explain some of the difficulties encountered by Tilman and Grime. This view is echoed by Goldberg and Novoplansky (1997) who state that the theories of Tilman and Grime provide explanations for some cases although not all.

9.1.2: *Metabolic Fingerprinting*

The results from the experiments suggest that FT-IR can be effective in detecting changes in the metabolome due to alterations in environmental parameters such as competition, enhanced UV-B and nutrient status of the soil. However, this study has highlighted problems involving the reproducibility of the technique. This discussion initially compares the results from all the experiments before discussing the problems identified in the context of other studies and to what extent metabolic fingerprinting can be used in systems biology.

There were many cases where changes in the metabolome were reflected in shifts in aboveground biomass. The metabolome of *Vaccinium myrtillus* was altered by UV-B (Figure 8.6) whilst none of the other sub-arctic shrubs were affected. This correlated to the fact that only *V. myrtillus* was affected by UV-B in terms of biomass (Figure 8.1). In the storage experiment (Chapter Three), UV-B only affected the flowering of *Lotus japonicus* and not biomass (Figure 3.7) which correlated to flowers being the only organs to show a metabolomic difference. *Agrostis tenuis*, the main determinant of community biomass in the artificial sub-montane community experiment (Chapter Seven), showed significant metabolomic alterations due to soil fertility (Figure 7.3) which correlated to the decrease in community biomass with decreased nutrients (Figure 7.2). This confirms the hypothesis that FT-IR can be used as a tool to detect chemical alterations due to changes in abiotic conditions.

There were also incidences where alterations were observed in the metabolome although not in growth parameters. In the mutant experiment (Chapter Six), the metabolome of *L. perenne* was different depending on whether it had been grown with the wild type or non-nodulating *L. japonicus* mutants (Figure 6.2). The two *L. japonicus* strains also showed chemical differences depending on whether it had been grown with the different strain (Figure 6.2). This was notable as *L. perenne* did not respond differently to the two strains of *L. japonicus*. This illustrates that FT-IR can be used to detect alterations in the environment due to biotic factors and that it is also sensitive to chemical changes that do not have a physical manifestation.

These results support the evidence that FT-IR is a valuable tool in detecting a broad range of chemical differences. Its efficacy in rapidly assess the chemical composition of leaves to determine soil fertility has been recognised by agricultural scientists (Dixon *et al.*, 2006; Yan *et al.*, 2006) and its potential to discriminate between different strains of *Triticum aestivum* to screen for allelopathic activity has also been suggested (Wu *et al.*, 2007). It has also been presented as a tool to detect whether there is substantial equivalence between transgenic and wild-type potatoes (Colquhoun *et al.*, 2006) and as a screening tool for phytomedically useful compounds (Urich-Merzenich *et al.*, 2007). These studies and the results from this thesis suggest that FT-IR is sufficiently sensitive to detect chemical differences in a variety of biological samples and can be used as an effective screening tool.

A potential disadvantage is that the numerous stages of the FT-IR process, from sample preparation to chemometric analysis, can introduce substantial experimental error. This problem has been identified by most researchers in metabolomics and

there has been much debate into how to minimise experimental error and the problems of reproducibility (Bino *et al.*, 2004; Jenkins *et al.*, 2004). Brown *et al.* (2004) described a concept called the 'metabolomics pipeline' whereby the process of experimental design, instrument optimization, data storage and manipulation leads to the incremental introduction of experimental error. Furthermore, Bamba and Fukusaki (2006) believed that the problems start before the FT-IR process and that the cultivation of living organisms, sampling and sample preparation are equally as important. There have been attempts to create an international standard to maximise reproducibility and allow for comparisons between different data sets (Jenkins *et al.*, 2004; Castle *et al.*, 2006) although they have been criticised (Fukusaki & Kobayashi, 2005; Ryan & Robards, 2006). Fukusaki and Kobayashi (2005) suggested that reproducibility was impossible in metabolic fingerprinting in plant physiology experiments as the multivariate analysis is sensitive to minor changes in the spectra. Therefore, whilst international standardisation may be successful for other disciplines, such as the MIAME method of microarray analysis (Brazma *et al.*, 2001), it is inappropriate for metabolic fingerprinting.

The critique of Fukusaki and Kobayashi (2005) also highlighted the importance of data interpretation. The technique was kept standard throughout the whole of the project with PCA being applied to the matrix with the first PC being analysed for treatment differences by ANOVA. In many ways this was fortuitous as spectral processing such as CO_2-peak removal and normalising between one and zero had no effect. Furthermore, the first PC was consistently above 95% allowing for one-way ANOVA as opposed to MANOVA which would have necessitated comparison of data sets analysed using different techniques. Furthermore, PCs other than the first

showed no differences and PC loading plots showed that there was not a wavelength that was principally responsible for differences. However, given the potential number of techniques that could have been used it is important that the statistical analysis is made clear if any form of international standardisation is to be achieved. In many ways this is hampered by the continual development of novel statistical methodologies (Seger & Sturm, 2007) such as genetic programming (Jarvis & Goodacre, 2004; Goodacre, 2005). Whilst these methodologies are highly successful and useful, they illustrate that metabolomics is still a developing area and attempts to standardise methodologies too soon will either become obsolete or hinder progress.

Despite the difficulties in reproducibility, it is clear that in the majority of cases, FT-IR can be used to detect environmental perturbations. Given that it provides a rapid methodology to find chemical differences it could then be used as a platform from which to base further studies. This has been successfully achieved by Kaderbhai *et al.* (2003) who noted differences in *Escherichia coli* secretions using FT-IR which justified the use of ESI-MS to pinpoint the specific chemical changes. Allwood *et al.* (2006) found that FT-IR showed differences in the metabolome of *Brachypodium distachyon* when infected by *Magnaporthe grisea*. In this case, the differences could be located in the fatty acid region of the spectra which was confirmed by ESI-MS. A similar approach in another pythopathology experiment was successfully achieved by Huang *et al.* (2006). However in all cases in this thesis, no wavelength contributed significantly to the loading of the PC, so it was not possible to determine which functional groups may have been contributing to the differences. However, it is clear that the results from the FT-IR justify further analysis using MS or NMR, especially

with both techniques being increasingly important in metabolomics (Mattioli *et al.*, 2006; Want *et al.*, 2007).

Another future aim of metabolic fingerprinting is in isolating biomarkers that can be used to detect whether certain environmental perturbations have occurred (Gidman *et al.*, 2004; 2005; 2006; Johnson *et al.*, 2003). In theory this method precludes the need to use MS as the biomarkers could be identified as peaks in the FT-IR spectrum. However, a drawback is that the signals used to differentiate between samples from one experiment can be the same as another. This was most evident in the glasshouse experiment (Chapter Four). This study compared the metabolome of monoculture and 50% mixture for *L. perenne* and *L. japonicus* roots and shoots at three densities (therefore twelve situations; Figure 4.4). In ten of the twelve situations, the monocultures were different from the mixtures which suggests that FT-IR can detect biochemical differences due to competition. However, signals from the monocultures and mixtures may have differed in one situation although the signal for the monoculture was often the same as a mixture in another. For example, monocultures and mixtures were clearly different for *L. perenne* shoots at low density (Figure 4.4). However, the signature for the mixture was the same as the monoculture at medium density (Figure 4.4). It is clear that changes due to competition are reflected in the metabolome although the signatures are not unique for each case ruling out the potential use of FT-IR to identify biomarkers.

The possibility that a biomarker may not be unique for each treatment has been highlighted by Fiehn (2001). This is of particular significance in plant physiology whereby identification of signals for a range of perturbations is one of the central

reasons for using this technique (Fukusaki & Kobayashi, 2005). This hypothesis rests on the assumption that a plant sample contains a "chemical log-book" which documents the nature of its environment. The scientific rationale is that a change to the environment, such as decreased nutrient availability, is reflected as a biochemical change (for example, lowered nitrogen content in poor nutrient conditions). The problematic assumption is that each alteration in the environment is reflected as a separate signal in the metabolome that can be then used as biomarkers. To test this assumption would necessitate testing all perturbations to show which biomarkers have similar signatures. This is clearly untenable and means work with biomarkers should be circumspect. This also has far-reaching implications as FT-IR has been used as a diagnostic tool in medicine (Harrigan *et al.*, 2004; Ellis & Goodacre, 2006; Winder *et al.*, 2006). This problem is also relevant to systems biologists who have the confounding factor of trying to integrate a variety of processes and organisms (Schauer & Fernie, 2006; Goodacre, 2007).

As a screening tool to discriminate between plant samples subjected to both abiotic and biotic stress, FT-IR is highly effective. This justifies the use of further metabolomic techniques such as ESI-MS to discover the chemical basis of the interference between *L. perenne* and *L. japonicus*. However, as a tool to obtain biomarkers, FT-IR is limited and further work is also needed to minimise experimental error. Nonetheless, metabolic fingerprinting is still a developing field and this study has shown that with further development, FT-IR has the potential to be of great use to post-genomic research.

9.1.2: *The Mechanistic Basis of Overyielding*

The second main conclusion from this project is that the facilitative effect between grasses and legumes may not be due to the nitrogen fixing capacity of the legumes. The traditionalist view was that the ability to fix nitrogen benefited companion species growing in the vicinity of the legumes. However, this thesis has consistently shown that the facilitative effect, at least in the early stages of an interaction, is due to complementarity of resource competition. This is in line with a number of revisionist sources that suggest facilitation is a common phenomenon and non-legume mixtures and that nitrogen-fixation is unlikely to explain changes observed in short-term experiments. This discussion integrates the results from all the relevant experiments and discusses the data in the context of the revisionist view.

All four experiments involving *L. perenne* and *L. japonicus* showed a facilitative effect at some point, whereby the mixtures were more productive than the monocultures. This was most clearly observed in the outdoor experiments. The facilitative effect was clear in all three harvests of the outdoor developmental experiment (Chapter Four; Figure 4.6) and in the first harvest of the UV-B experiment (Chapter Five, Figure 5.1). The effect was less clearly observed in the second and third harvests of the UV-B experiment as UV-B and UV-A treatment differences were beginning to show although there was still clearly no antagonistic effect (whereby the mixtures were smaller than both monocultures). The same can be said of the glasshouse experiment (Figure 4.3) and mutant experiment (Figure 6.1) where it was clear that a mechanism other than antagonism was occurring. This confirms the

widely observed phenomenon that mixtures of grasses and legumes will overyield (Menchaca & Connolly, 1990; Niang *et al.*, 1998; Springer, 2001).

However, it was still apparent that *L. perenne* and *L. japonicus* were still competing with each other and having a negative effect. In every experiment the interspecific competition coefficients (and intraspecific coefficients) were always negative. In no case was there a positive coefficient which would be hypothesised should the legume be directly benefiting the grass. The substitution rates when calculated (they sometimes could not be calculated as the interspecific effect was insignificant) were consistently positive which is indicative of two competing species (the quotient of two negative values is always positive). However, the fact the intraspecific coefficients were always bigger than the interspecific coefficients explains the seemingly beneficial effect of growing two species together that are antagonistic at the individual level. This is because competition is not as intense as the monocultures, presumable because of competition for the same resource, and thus the mixtures, whilst still competing, have a lowered competitive pressure.

This suggests that nitrogen-fixation is not responsible for the facilitative effect in these experiments. This was most clearly observed in the mutant experiment whereby non-nodulating and non-mycorrhizal *L. japonicus* did not respond differently to the wild-types in competition despite the wild type nodulating (Chapter Six). There is also growing evidence from other sources that the role of nitrogen-fixation has been overestimated (van Ruijven & Berendse, 2003) and that most plant mixtures will overyield (Putnam & Allen, 1992; Ayisi *et al.*, 1997; Skelton & Barrett, 2005). Results from ecosystem models universally predict increased biomass with species

richness (Hooper & Dukes, 2004; Lambers *et al.*, 2004) which corroborates with the multiple regression models gained from these studies. This view is contrary to the traditional view that hypothesised the role of nitrogen-fixation as the main cases of facilitation (Dupraz *et al.*, 1998; Li *et al.*, 2003; Rodriguez-Echeverria *et al.*, 2003). However, there have been surprisingly very few studies that have directly tested this. Studies using N-15 labelling showed significant exchange of nitrogen between different legumes and grasses although it was unclear whether this benefited the grass or not (Høgh-Jensen & Schjoerring, 2000; Høgh-Jensen, 2006). Therefore, this thesis supports the view that nitrogen-fixation may not be as important as previously assumed.

Future work should continue using nitrogen-fixing mutants in other species to test the new hypothesis that nitrogen-fixing is not important in plant interference. N-15 labelling is a powerful tool although it should be done in conjunction with an extensive plant competition experiment to ascertain whether nitrogen-sharing is having an effect or not. A large multi-species pairwise response-surface design including nitrogen-fixing mutants and N-15 labelling would be highly desirable in testing this hypothesis.

9.2: General Conclusion

This study found that, in the short-term, nitrogen fixation is unlikely to be the reason for the facilitative effects between *Lolium perenne* and *Lotus corniculatus*. It is more probable that the reason for overyielding was due to decreased intraspecific competition which is more intense.

The study also showed that high levels of UV-B had an impact on plant competition. It is likely that broad-leaved species are likely to be damaged resulting in a predominance of thin-leaved species or those more adapted to elevated UV-B radiation. However, it is unlikely that natural levels of UV-B will have a great effect on natural communities. In terms of the theories of Tilman and Grime, the effects of UV-B support Grime's theory in that competition was eliminated under high conditions of stress although not all the results supported this.

Metabolic fingerprinting was shown to be an effective tool in detecting chemical changes due to competition and UV-B stress. However, the complexity of the interactions means that metabolic fingerprinting may not be suitable for the identification of biomarkers.

9.3: Thesis Summary

1. The facilitative effect between *Lolium perenne* and *Lotus corniculatus* or *Lotus corniculatus* var. *japonicus* is probably due to decreased intraspecific competition and not due to nitrogen fixation in the short-term
2. Enhanced UV-B alters the competitive balance between two species in favour of species more tolerant of UV-B
3. The effects of UV-B are more noticeable under high nutrient conditions suggesting that communities exposed to enhanced UV-B and nitrogen deposition are most at risk
4. FT-IR can be used to screen for biochemical changes induced by biotic and abiotic stress thus forming an effective platform from which to base other metabolomic methodologies

9.4: Future Work

It was concluded that overyielding in mixtures of *Lolium perenne* and *Lotus corniculatus* was due to the fact that competitive pressure was higher in monocultures than in mixtures. For this reason the mixtures had a greater biomass than the pure stands which consequently resulted in the facilitative effect. Future work could determine whether this mechanism is responsible for the facilitative effect observed in other grass-legume mixtures. A variety of pairwise species mixtures using different species would help confirm this. This could also be also tested by using a variety of different legume mutants incapable of forming symbiotic relationships. Moreover, all the experiments in this thesis were short-term and it is possible that nitrogen-fixation could alter a competitive interaction over a longer period of time. For this reason, long-term studies using similar species would be desirable.

UV-B was shown to be an effective tool in assessing the effects of stress on plant competition and could play a valuable role in evaluating the Tilman-Grime debate. These results point towards Grime's theory that competitive effects are lessened under stress although there were cases, as with the sub-montane community experiment, where competition was unimportant under both stress and control conditions. This suggests that both theories may be relevant under certain conditions and for different species. It is clear that more extensive empirical work is needed to test these theories. It is therefore recommended that a large-scale experiment, using many different species and functional types, is set up to investigate whether enhanced UV-B reduces competitive ability.

It was also clear from the sub-montane community experiment that enhanced UV-B interacted with nutrient availability in determining community biomass. This has important ramifications for studies concerned with modelling the effects of climate change such as nitrogen deposition and ozone depletion. It could therefore be hypothesised that increased nitrogen pollution would make plant communities more susceptible to enhanced UV-B should ozone depletion decrease. In order to test this hypothesis, an outdoor experiment using artificial mesocosms of a variety of plant communities would be of great use. An experimental set-up, such as the Penglais Farm UV-B site with communities designed using response surface analysis, was shown to be effective and could be used for this purpose.

Metabolic fingerprinting by FT-IR was shown to be highly effective in detecting chemical perturbations induced by both competition and abiotic stress. However, in order to determine what the exact chemical changes were it is necessary to employ further technologies such as MS. By using MS, it would be clear what the precise chemicals are that are responsible for alterations to the metabolome. In the case of enhanced UV-B it could be hypothesised that flavonoids would be responsible given the large body of evidence to suggest that these chemicals are the most important in UV-B defence. The chemical alterations induced by competition could also be discovered. Therefore, FT-IR has shown that chemical changes are induced by competition and present in soil leachates; the next step is to use MS to discover what the precise changes are.

References

Acosta L.R. & Evans W.F.J. (2000). Design of the Mexico City UV monitoring network: UV-B measurements at ground level in the urban environment. *Journal of Geophysical Research – Atmospheres* **105**, 5017-5026.

Aerts R. (1999). Interspecific competition in natural plant communities: Mechanisms, trade-offs and plant-soil feedbacks. *Journal of Experimental Botany* **50**, 29-37.

Aerts R., Berendse F., De Caluwe H. & Schmitz M. (1990). Competition in heathland along an experimental gradient of nutrient availability. *Oikos* **57**, 310-318.

Albert K.R., Mikkelsen T.N. & Ro-Poulsen H. (2005). Effects of ambient versus reduced UV-B radiation on high arctic *Salix arctica* assessed by measurements and calculations of chlorophyll a fluorescence parameters from fluorescence transients. *Physiologia Plantarum* **124**, 208-226.

Allen D.J., Nogués S., Morison J.I.L., Greenslade P.D., McLeod A.R. & Baker N.R. (1999). A thirty percent increase in UV-B has no impact on photosynthesis in well-watered and droughted pea plants in the field. *Global Change Biology* **5**, 235-244.

Allwood J.W., Ellis D.I., Heald J.K., Goodacre R. & Mur L.A.J. (2006). Metabolomic approaches reveal that phosphatidic and phosphatidyl glycerol phospholipids are major discriminatory non-polar metabolites in responses by *Brachypodium distachyon* to challenge *Magnaporthe grisea*. *The Plant Journal* **46**, 351-368.

Alonso I. & Hartley S.E. (1998). Effect of nutrient supply, light availability and herbivory on the growth of heather and three competing grass species. *Plant Ecology* **137**, 203-212.

Aphalo P.J. (2003). Do current levels of UV-B radiation affect vegetation? The importance of long-term experiments. *New Phytologist* **160**, 273-276.

Aphalo P.J., Tegelberg R. & Julkunen-Tiitto (1999). The modulated UV-B irradiation system at the University of Joensuu. *Biotronics* **28**, 109-120.

Arias M.E., Gonzalez-Perez J.A., Gonzalez-Vila F.J. & Ball A.S. (2005). Soil health – a new challenge for microbiologists and chemists. *International Microbiology* **8**, 13-21.

Arvanitoyannis I. & Nikolaos T. (2005). Implementation of quality control methods in conjunction with chemometrics toward authentication of dairy products. *Critical Reviews in Food Science and Nutrition* **45**, 231-249.

Austin M.P., Fresco L.F.M., Nicholls A.O., Groves R.H. & Kaye P.E. (1988). Competition and relative yield: Estimation and interpretation at different densities and under various nutrient concentrations using *Silybum marianum* and *Cirsium vulgare*. *Journal of Ecology* **76**, 157-171.

Avery L.M., Smith R.I.L. & West H.M. (2003). Response of rhizosphere microbial communities associated with Antarctic Hairgrass (*Deschampsia antarctica*) to UV radiation. *Polar Biology* **26**, 525-529.

Avery L.M., Thorpe P.C., Thompson K., Paul N.D., Grime J.P. & West H.M. (2004). Physical disturbance of an upland grassland influences the impact of elevated UV-B radiation on metabolic profiles of below-ground micro-organisms. *Global Change Biology* **10**, 1146-1154.

Ayisi K.K., Putnam D.H., Vance C.P., Russelle M.P. & Allan D. (1997). Strip intercropping and nitrogen effects on seed, oil, and protein yields of canola and soybean. *Agronomy Journal* **89**, 23-29.

Baddeley J.A., Thompson D.B.A. & Lee J.A. (1994). Regional and historical variation in the nitrogen content of *Racomitrum lanuginosum* in Britain in relation to atmospheric nitrogen deposition. *Environmental Pollution* **84**, 189 – 196.

Bais A.F., Zerefos C.S., Meleti C., Ziomas I.C. & Tourpali K. (1993). Spectral measurements of solar UVB radiation and its relations to ozone, SO2 and clouds. Journal of Geophysical Research – Atmospheres **98**, 5199-5204.

Ballaré C.L. & Scopel A.L. (1997). Phytochrome signalling in plant canopies: Testing its population-level implications with photoreceptor mutants of *Arabidopsis*. *Functional Ecology* **11**, 441-450.

Ballaré C.L., Scopel A.L. & Mazza C.A. (1999). Effects of solar UV-B radiation on terrestrial ecosystems: Case studies from southern South America. In: Stratospheric Ozone Depletion. The effects of enhanced UV-B radiation on terrestrial ecosystems (Ed. J. Rozema), pp 293-311. Backhuys Publishers, Leiden.

Bamba T. & Fukusaki E. (2006). Technical problems and practical operations in plant metabolomics. *Journal of Pesticide Science* **31**, 300-304.

Barker C.G., Power S.A., Bell J.N.B. & Orme C.D.L. (2004). Effect of habitat management on heathland response to atmospherics nitrogen deposition. *Biological Conservation* **120**, 41-52.

Barnes P.A., Ballaré C.L. & Caldwell M.M. (1996). Photomorphogenic effects of UV-B radiation on plants: Consequences for light competition. *Journal of Plant Physiology* **148**, 15-20.

Barnes P.A., Beyschlag W., Ryel R., Flint S.D. & Caldwell M.M. (1990a). Plant competition for light analyzed with a multispecies canopy model. 3. Influence of canopy structure in mixtures and monocultures of wheat and wild oat. *Oecologia* **82**, 560-566.

Barnes P.W., Flint S.D. & Caldwell M.M. (1990b). Morphological responses of crop and weed species of different growth forms to ultraviolet-B radiation. *American Journal of Botany* **77**, 1354-1360.

Barnes P.W., Flint S.D. & Caldwell M.M. (1995). Early-season effects of supplemented solar UV-B radiation on seedling emergence, canopy structure, simulated stand and photosynthesis and competition for light. *Global Change Biology* **1**, 43-53.

Barnes P.W., Maggard S., Holman S.R. & Vergara B.S. (1993). Intraspecific variation in sensitivity to UV-B radiation in rice. *Crop Science* **33**, 1041-1046.

Bauer G. & Richter W. (1996). *Optical characterization of epitaxial semiconductor layers*, pp 225-232. Springer-Verlag, Berlin.

Beckage B. & Gross L.J. (2006). Overyielding and species diversity: what should we expect? *New Phytologist* **172**, 140-148.

Belcher J.W., Keddy P.A. & Twolan-Strutt L. (1995). Root and shoot competition intensity along a soil depth gradient. *Journal of Ecology* **83**, 673-682.

Berendse F. & Aerts R. (1984). Competition between *Erica tetralix* L. and *Molinia caerulea* (L.) Moench as affected by the availability of nutrients. *Acta Oecologia – Oecologia Plantarum* **5**, 3-14.

Bino R.J., Hall R.D., Fiehn O., Kopka J., Saito K., Draper J., Nikolau B.J., Mendes P., Roessner-Tunali U., Beale M.H., Tretheway R.N., Lange B.M., Wurtele E.S. & Sumner L.W. (2004). Potential of metabolomics as a functional genomics tool. *Trends in Plant Science* **9**, 418-425.

Bjerke J.W., Zielke M. & Solheim B. (2003). Long-term impacts of simulated climate change on secondary metabolism, thallus structure and nitrogen fixation activity in two cyanolichens from the Arctic. *New Phytologist* **159**, 361-367.

Björn L.O. & Murphy T.M. (1985). Computer calculation of solar ultraviolet-radiation at ground level. *Physiologie Vegetale* **23**, 555-561.

Björn L.O., Callaghan T.V., Johnsen I., Lee J.A., Manetas Y., Paul N.D., Sonesson M., Wellburn A.R., Coop D., Heide-Jørgensen H.S., Gehrke C., Gwynn Jones D., Johanson U., Kyparissis A., Levizou E., Nikopoulos D., Petropoulou Y. & Stephanou M. (1997). The effects of UV-B radiation on European heathland species. *Plant Ecology* **128**, 252-264.

Bobbink R, Bik L. & Willems J.H. (1998b). Effects of nitrogen fertilization on vegetation structure and dominance of *Brachypodium pinnatum* (L.) Beauv. in chalk grassland. *Acta Botanica Neerlandica* **37**, 231-242.

Bobbink R. & Willems J.H. (1991). Impact of different cutting regimes on the performance of *Brachypodium pinnatum* in Dutch chalk grassland. *Biological Conservation* **56**, 1-21.

Bobbink R., Hornung M. & Roelofs J.G.M. (1998a). The effects of air-borne nitrogen pollutants on species diversity in natural and semi-natural European vegetation. *Journal of Ecology* **86**, 717-738.

Boelen P., de Boer M.K., de Bakker N.V.J & Rozema J. (2006). Outdoor studies on the effects of solar UV-B on bryophytes: Overview and methodology. *Plant Ecology* **182**, 137-152.

Boorman L.A. & Fuller R.M. (1982). Effects of added nutrients on dune swards grazed by rabbits. *Journal of Ecology* **70**, 345-355.

Bouwman A.F., van Drecht G. & van der Hoek K.W. (2005). Global and regional surface nitrogen balances in intensive agricultural production systems for the period 1970-2030. *Pedosphere* **15**, 137-155.

Brazma A., Hingamp P., Quackenbush J., Sherlock G., Spellman P., Stoeckert C., Aach J., Ansorge W., Ball C.A., Causton H.C., Gaasterland T., Glenisson P., Holstege F.C.P., Kim I.F., Markowitz V., Matese J.C., Parkinson H., Robinson A., Sarkans U., Schulze-Kremer S., Stewart J., Taylor R., Vilo J. & Vingron M. (2001). Minimum information about a microarray experiment (MIAME) – towards standards for microarray data. *Nature Genetics* **29**, 365-371.

Bredahl L., Ro-Poulsen H. & Mikkelsen T.N. (2004). Reduction of the ambient UV-B radiation in the high-Arctic increases F_V/F_M in *Salix arctica* and *Vaccinium uliginosum* and reduces stomatal conductance and internal CO_2 concentration in *Salix arctica*. *Arctic, Antarctic & Alpine Research* **36**, 364-369.

Brereton R.G. (1987). Chemometrics in Analytical Chemistry: A Review. *Analyst* **112**, 1635-1657.

Brewer J.S., Rand T., Levine J.M. & Bertness M.D. (1998). Biomass allocation, clonal dispersal, and competitive success in three salt-marsh plants. *Oikos* **82**, 347-353.

Britto D.T. & Kronzucker H.J. (2002). $NH4^+$ toxicity in higher plants: a critical review. *Journal of Plant Physiology* **159**, 567-584.

Britton A., Marrs R., Pakeman R. & Carey P. (2003). The influence of soil-type, drought and nitrogen addition on interactions between *Calluna vulgaris* and *Deschampsia flexuosa*: Implications for heathland regeneration. *Plant Ecology* **166**, 93-105.

Brooker R.W. (2006). Plant-plant interactions and environmental change. *New Phytologist* **171**, 271-284.

Brown M., Dunn W.B., Ellis D.I., Goodacre R., Handl J., Knowles J.D., O'Hagan S., Spasić I. & Kell D.B. (2004). A metabolome pipeline: From concept to data to knowledge. *Metabolomics* **1**, 39-51.

Bruce W.H., Anderson J.H., Toohey D.W., Fahey D.W., Kawa S.R., Jones R.L., McKenna L.R. & Poole L.R. (1991). The potential for ozone depletion in the Arctic polar stratosphere. *Science* **252**, 1260-1266.

Bruck H. & Guo S.W. (2006). Influence of N form on growth and photosynthesis of *Phaseolus vulgaris* L. plants. *Journal of Plant Nutrition & Soil Science – Zeitschrift fur Pflanzenernahrung und Bodenkunde* **169**, 849-856.

Bruno J.F., Stachowicz J.J. & Bertness M.D. (2003). Inclusion of facilitation into ecological theory. *Trends in Ecology & Evolution* **18**, 119-125.

Bullock J.M., Mortimer A.M. & Begon M. (1994). The effect of clipping on interclonal competition in the grass *Holcus lanatus* – a response-surface analysis. *Journal of Ecology* **82**, 259-270.

Caldwell M.M. & Flint S.D. (1997). Uses of biological spectral weighting functions and the need of scaling for the ozone reduction problem. *Plant Ecology* **128**, 66-76.

Caldwell M.M. (1971). Solar ultraviolet radiation and the growth and development of higher plants. *Photophysiology* **6**: 131-177

Caldwell M.M., Flint S.D. & Searles P.S. (1994). Spectral balance and UV-B sensitivity of Soybean – a field experiment. *Plant Cell & Environment* **17**, 267-276.

Caldwell M.M., Gold W.G., Harris G. & Ashurst C.W. (1983). A modulated lamp system for solar UV-B (280-320 nm) supplementation studies in the field. *Photochemistry and Photobiology* **37,** 479-485.

Caldwell M.M., L.B. Camp C.W. Warner & S.D. Flint (1986). Action spectra and their key role in assessing biological consequences of solar UV-B radiation change. In: Worrest, R.C. and M.M. Caldwell (Eds.): Stratospheric ozone reduction, solar ultraviolet radiation and plant life. Springer-Verlag, Berlin. P.87-111.

Campbell B.D. & Grime J.P. (1992). An experimental test of plant strategy theory. Ecology 73, 15-29.

Campbell B.D., Grime J.P., Mackey J.M.L. & Jalili A. (1991). The quest for a mechanistic understanding of resource competition in plant communities – the role of experiments. *Functional Ecology* **5**, 241-253.

Carroll J.A., Caporn S.J.M., Cawley L., Read D.J. & Lee J.A. (1999). The effects of increased deposition of atmospheric nitrogen on Calluna vulgaris in upland Britain. *New Phytologist* **141**, 423-431.

Carter M.S. & Ambus P. (2006). Biologically fixed N_2 as a source of N_2O production in a grass-clover mixture, measured by N-15(2). *Nutrient Cycling in Agroecosystems* **74**, 13-26.

Castle A.L., Fiehn O., Kaddurah-Daouk R. & Lindon J.C. (2006). Metabolomics Standards Workshop and the development of international standards for reporting metabolomics experimental results. *Briefing in Bioinformatics* **7**, 159-165.

Chabot S., Bel-Rhlid R., Chenevert R. and Piché Y. (1992). Hyphal growth promotion in vitro of the VA mycorrhiza fungus *Gigaspora margarita* Becker & Hall,

by the activity of structurally specific flavonoid compounds under CO_2-enriched conditions. *New Phytologist* **122**, 461-467

Chimphango S.B.M., Musil C.F. & Dakora F.D. (2003a). Effects of UV-B radiation on plant growth, symbiotic function and concentration of metabolites in three tropical grain legumes. *Functional Plant Biology* **30**, 309-318.

Chimphango S.B.M., Musil C.F. & Dakora F.D. (2003b). Response of purely symbiotic and NO_3-fed nodulated plants of *Lupinus luteus* and *Vivia atropurpurea* to ultraviolet-B radiation. *Journal of Experimental Botany* **54**, 1771-1784.

Chimphango S.B.M., Musil C.F. & Dakora F.D. (2004). Responses to ultraviolet-B radiation by purely symbiotic and NO_3 fed nodulated tree and shrub legumes indigenous to southern Africa. *Tree Physiology* **24**, 181-192.

Chu G.X., Shen Q.R. & Cao J.L. (2004). Nitrogen fixation and N transfer from peanut to rice cultivated in aerobic soil in an intercropping system and its effect on soil N fertility. *Plant & Soil* **263**, 17-27.

Colquhoun I.J., Le Gall G., Elliott K.A., Mellon F.A. & Michael A.J. (2006). Shall I compare thee to a GM potato? *Trends in Genetics* **22**, 525-528.

Connolly J. & Wayne P. (1996). Asymmetric competition between plant species. *Oecologia* **108**, 311-320.

Connolly J. & Wayne P. (2005). Assessing determinants of community biomass composition in two-species plant competition studies. *Oecologia* **142**, 450-457.

Connolly J. (1986). On difficulties with replacement-series methodology in mixture experiments. *Journal of Applied Ecology* **23**, 125-137.

Connolly J., Goma H.C. & Rahim K. (2001a). The information content indicators in intercropping research. *Agriculture Ecosystems & Environment* **87**, 191-207.

Connolly J., Wayne P. & Bazzaz F.A. (2001b). Inter-specific competition in plants: How well do current methods answer fundamental questions? *American Naturalist* **157**, 107-125.

Connolly J., Wayne P. & Murray R. (1990). Time course of plant-plant interactions in experimental mixtures of annuals – density, frequency, and nutrient effects. *Oecologia* **82**, 513-526.

Cooley N.M., Truscott H.M.F., Holmes M.G. & Attridge T.H. (2000). Outdoor ultraviolet polychromatic action spectra for growth responses of *Bellis perennis* and *Cynosurus cristatus*. *Journal of Photochemistry & Photobiology B – Biology* **59**, 64-71.

Correia C.M., Areal E.L.V., Torres-Pereira M.S. & Torres-Pereira J.M.G. (1998). Intraspecific variation in sensitivity to ultraviolet-B radiation in maize grown under

field conditions. I. Growth and morphological aspects. *Field Crops Research* **59**, 81-89.

Correia C.M., Pereira J.M.M., Coutinho J.F., Björn L.O. & Torres-Pereira J.M.G. (2005). Ultraviolet-B radiation and nitrogen affect the photosynthesis of maize: A Mediterranean field study. *European Journal of Agronomy* **22**, 337-347.

Costa C.S.B., Marangoni J.C. & Azevado A.M.G. (2003). Plant zonation in irregularly flooded salt marshes: Relative importance of stress tolerance and biological interactions. *Journal of Ecology* **91**, 951-965.

Craine J.M. (2005). Reconciling plant strategy theories of Grime and Tilman. *Journal of Ecology* **93**, 1041-1052.

Creelman R.A. & Mullet J.E. (1995). Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences* **92**, 4114-4119.

Dai Q.J., Coronel V.P., Vergara B.S., Barnes P.W. & Quintos A.T. (1992). Ultraviolet-B radiation effects on growth and physiology of 4 rice cultivars. *Crop Science* **32**, 1269-1274.

Dai Q.J., Peng S.B., Chavez A.Q. & Vergara B.S. (1994). Intraspecific responses of 188 rice cultivars to enhanced UV-B radiation. *Environmental & Experimental Botany* **34**, 433-442.

Davis M.A., Wrage K.J. & Reich P.B. (1998). Competition between tree seedlings and herbaceous vegetation: Support for a theory of resource supply and demand. *Journal of Ecology* **86**, 652-661.

Day T.D., Ruhland C.T. & Xiong F.S. (2001). Influence of solar ultraviolet radiation on Antarctic terrestrial plants: Results from a four-year field study. *Journal of Photochemistry & Photobiology B: Biology* **62**, 78-87.

De Kroon H. & Bobbink R. (1997). Clonal plant performance under elevated nitrogen deposition with special reference to *Brachypodium pinnatum* in chalk grassland. *The Ecology and Evolution of Clonal Plants* (Eds: H. De Kroon & J. Van Groenendael), pp. 359-379. Backhuys Publishers, Leiden.

De La Rosa T.M., Aphalo P.J. & Lehto T. (2003). Effects of ultraviolet-B radiation on growth of mycorrhizas and mineral nutrition of silver birch (*Betula pendula* Roth.) seedlings in low nutrient conditions. *Global Change Biology* **9**, 65-73.

De La Rosa T.M., Julkunen-Tiito R., Lehto T. & Aphalo P.J. (2001). Secondary metabolites and nutrient concentrations in silver birch seedlings under five levels of daily UV-B exposure and two relative nutrient addition rates. *New Phytologist* **150**, 121-131.

Debain S., Curt T. & Lepart J. (2005). Indirect effects of grazing on the establishment of *Pinus sylvestris* and *Pinus nigra* seedlings in calcareous grasslands in relation to resource level. *Ecoscience* **12**, 192-201.

Deckmyn G. & Impens I. (1997). Combined effects of enhanced UV-B radiation and nitrogen deficiency on the growth, composition and photosynthesis of rye (*Secale cereale*). *Plant Ecology* **128**, 235-240.

Degünther M., Meerkötter R., Albold A. & Seckmeyer G. (1998). Case study on the influence of inhomogeneous surface albedo on UV irradiance. *Geophysical Research Letters* **25**, 3587-3590.

Ditommaso A. & Aarsen L.W. (1991). Effect of nutrient level on competition intensity in the field for 3 coexisting grass species. *Journal of Vegetation Science* **2**, 513-522.

Dixon R.A., Gang D.R., Charlton A.J., Fiehn O., Kuiper H.A., Reynolds T.L., Tjeerma R.S., Jeffery E.H., German J.B., Ridley W.P & Seiber J.N. (2006). Perspectives – Applications of metabolomics in agriculture. *Journal of Agricultural & Food Chemistry* **54**, 8984-8994.

Dodd M.E., Silvertown J., McConway K., Potts J. & Crawley M. (1994). Application of the British national vegetation classification to the communities of the Park Grass experiment through time. *Folia Geobotanica and Phytotaxonomica* **29**, 321-334.

Döhring T., Köfferlein M., Thiel S. & Seidlitz H.K. (1996). Spectral shaping of artificial UVB irradiation for vegetation stress research. *Journal of Plant Physiology* **148**, 115–119.

Doyle J.J. & Dickson E.E. (1987). Preservation of plant samples for DNA restriction endonuclease analysis. *Taxon* **36**, 715-722.

Dueck T.A., Dorel F.G., Ter Horst R. & van der Eerden L.J. (1990). Effects of ammonia sulphate and sulphur dioxide on frost sensitivity of Scots Pine (*Pinus sylvestris* L.). *Water, Air and Soil Pollution* **54**, 35-49.

Dukes J.S. & Mooney H.A. (1999). Does global change increase the success of biological invaders? *Trends in Ecology & Evolution* **14**, 135-139.

Dunn J.L. & Robinson S.A. (2002). Ultraviolet-B screening potential is increased in two cosmopolitan species than in a co-occurring Antarctic endemic moss: Implications of continuing ozone depletion. *Global Change Biology* **12**, 2282-2296.

Dupraz C., Simorte V., Dauzat M., Bertoni G., Bernadac A. & Masson P. (1998). Growth and nitrogen status of young walnuts as affected by intercropped legumes in a Mediterranean climate. *Agroforestry Systems* **43**, 71-80.

Ellis D.I. & Goodacre R. (2006). Metabolic fingerprinting in disease diagnosis: Biomedical applications of infrared and Raman spectroscopy. *Analyst* **131**, 875-885.

Ellis D.I., Broadhurst D., Kell D.B., Rowland J.J., Goodacre R. (2002). Rapid and quantitative detection of the microbial spoilage of meat by Fourier Transform Infrared spectroscopy and machine learning. *Applied and Environmental Microbiology* **68**, 2822-2828.

Ellis D.I., Harrigan G.G. & Goodacre R. (2003). Metabolomic fingerprinting with Fourier-transform infrared spectroscopy. In *Metabolic Profiling: its role in biomarker discovery and gene function analysis*, pp 111-124. Edited by Harrigan G.G & Goodacre R.G. Kluwer Academic Publishers.

Fan F.L., Zhang F.S., Song Y.N., Sun J.H., Bao X.G., Guo T.W. & Li L. (2006). Nitrogen fixation of faba bean (*Vicia faba* L.) interacting with a non-legume in two contrasting intercropping systems. *Plant & Soil* **283**, 275-286.

Fangmeier A., Hadwiger-Fangmeier A., van der Eerden L. & Jager H.J. (1994). Effects of atmospheric ammonia on vegetation – a review. *Environmental Pollution* **86**, 43-82.

Fargione J. & Tilman D. (2006). Plant species traits and capacity for resource reduction predict yield and abundance under competition in nitrogen-limited grassland. *Functional Ecology* **20**, 533-540.

Fargione J., Tilman D., Dybzinski R., Lambers J.H.R., Clark C., Harpole W.S., Knops J.M.H., Reich P.B. & Loreau M. (2007). From selection to complementarity: Shifts in the causes of biodiversity-productivity relationships in a long-term biodiversity experiment. *Proceedings of the Royal Society B – Biological Sciences* **274**, 871-876.

Farman J.C., Gardine B.G. & Shanklin J.D. (1985). Large losses of total ozone in Antarctica reveal seasonal ClO_X/NO_X interaction. *Nature* **315**, 207-210.

Feldheim K. & Connor J.K. (1995). The effects of increased UV-B radiation on growth, pollination success and lifetime female fitness in two *Brassica* species. *Oecologia* **106**, 284-297.

Fiehn O. (2001). Combining genomics, metabolome analysis, and biochemical modelling to understand metabolic networks. *Comparative and Functional Genomics* **2**, 155-168.

Fiehn O. (2002). Metabolomics – the link between genotypes and phenotypes. *Plant Molecular Biology* **48**, 155-171.

Fiehn O., Kloska S. & Altmann T. (2001). Integrated studies on plant biology using multiparallel techniques. *Current Opinion in Biotechnology* **12**, 82-86.

Fiehn O., Kopka J., Dörmann P., Altmann T., Trethewey R.M. & Willmitzer L. (2000). Metabolite profiling for plant functional genomics. *Nature Biotechnology* **18**, 1157-1161.

Firbank L.G. & Watkinson A.R. (1985). On the analysis of competition within two-species mixtures of plants. *Journal of Applied Ecology* **22**, 308-317.

Fowler N.L. (1995). Density-dependent demography in 2 grasses – a 5 year study. *Ecology* **76**, 2145-2164.

Fox F.M. & Caldwell M.M. (1978). Competitive interaction in plant populations exposed to supplementary ultraviolet-B radiation. *Oecologia* **36**, 173-190.

Fraser L.H. & Keddy P.A. (2005). Can competitive ability predict structure in experimental plant communities? *Journal of Vegetation Science* **16**, 571-578.

Freckleton R.P. & Watkinson A.R. (2001). Predicting competition coefficients for plant mixtures: Reciprocity, transitivity and correlations with life-history traits. *Ecology Letters* **4**, 348-357.

Frederick J.E. & Snell H.E. (1988). Ultraviolet radiation levels during the Antarctic spring. *Science* **241**, 438-440.

Fukusaki E. & Kobayashi A. (2005). Plant metabolomics: Potential for Practical Operation. *Journal of Bioscience & Bioengineering* **100**, 347-354.

Furness N.H. & Upadhyaya M.K. (2002). Differential susceptibility of agricultural weeds to ultraviolet-B radiation. *Canadian Journal of Plant Science* **82**, 789-796.

Furness N.H., Joliffe P.A. & Upadhyaya M.K. (2005a). Competitive interactions in mixtures of broccoli and *Chenopodium album* grown at two UV-B radiation levels under glasshouse conditions. *Weed Research* **45**, 449-459.

Furness N.H., Joliffe P.A. & Upadhyaya M.K. (2005b). Ultraviolet-B radiation and plant communities: Experimental approaches and underlying mechanisms. *Photochemistry & Photobiology* **81**, 1026-1037.

Gallo M.E., Lauber C.L., Cabaniss S.E., Waldrop M.P., Sinsabaugh R.L. & Zak D.R. (2005). Soil organic matter and litter chemistry response to experimental N deposition in northern temperate deciduous forest ecosystems. *Global Change Biology* **11**, 1514-1521.

Galloway J.N., Denterre F.J., Capone D.G., Boyer E.W., Howarth R.W., Seitzinger S.P., Asner G.P., Cleveland C.C., Green P.A., Holland E.A., Karl D.M., Michaels A.F., Porter J.H., Townsend A.R. & Vorosmarty C.J. (2005). Nitrogen cycles: past, present and future. *Biogeochemistry* **70**, 153-226.

Galloway J.N., Dianwu Z., Thompson V.E. & Chong L.H. (1996). Nitrogen mobilisation in the United States of America and People's Republic of China. *Atmospheric Environment* **30**, 1551-1561.

Galloway J.N., Levy H. & Kashibhatha P.S. (1994). Year 2020 – Consequences of population-growth and development on deposition of oxidised nitrogen. *Ambio* **23**, 120-123.

Galloway J.N., Schlesinger W.H., Levy II H., Michaels A. & Schnoor J.L. (1995). Nitrogen fixation: Anthropogenic enhancement-environmental response. *Global Biogeochemical Cycles* **9**, 235-252.

Gaucherand S., Liancourt P. & Lavorel S. (2006). Importance and intensity of competition along a fertility gradient and across species. *Journal of Vegetation Science* **17**, 455-464.

Gehrke C., Johanson U., Callaghan T.V., Chadwick D. & Robinson C.H. (1995). The impact of enhanced ultraviolet-B radiation on litter quality and decomposition processes in Vaccinium leaves from the sub-Arctic. *Oikos* **72**, 213-222.

Gibson D.J., Connolly J., Hartnett D.C. & Weidenhamer J.D. (1999). Designs for greenhouse studies of interactions between plants. *Journal of Ecology* **87**, 1-16.

Gidman E., Goodacre R., Emmett B., Smith A.R. & Gwynn-Jones D. (2003). Investigating plant-plant interference by metabolomic fingerprinting. *Phytochemistry* **63**, 705-710.

Gidman E.A., Goodacre R., Emmett B., Shappard L.J., Leith I.D. & Gwynn Jones D. (2004). Applying metabolic fingerprinting to ecology: The use of Fourier-Transform Infrared Spectroscopy for the rapid screening of plant responses to N. *Water, Air & Soil Pollution* **4**, 251-258.

Gidman E.A., Goodacre R., Emmett B., Wilson D.B., Carroll J.A., Caporn J.M., Cresswell N. & Gwynn Jones D. (2005). Metabolic fingerprinting for bio-indication of nitrogen responses in *Calluna vulgaris* heath communities. *Metabolomics* **1**, 279-285.

Gidman E.A., Stevens C.J., Goodacre R., Broadhurst D., Emmett B. & Gwynn-Jones D (2006). Using metabolic fingerprinting of plants for evaluating nitrogen deposition impacts on the landscape level. *Global Change Biology* **12**, 1460-1465.

Gleason J.E., Hartia P.K., Herman J.R., Peteus R., Newman R., Stolarski R.S., Flynn L., Loabow G., Larko D., Seftor C., Wellemeyer C., Komhry W.D., Miller A.J. & Planet W. (1993). Record low global ozone in 1992. *Science* **290**, 323-526.

Gold W.G. & Caldwell M.M. (1983). The effects of ultraviolet-B radiation on plant competition in terrestrial ecosystems. *Physiologia Plantarum* **58**, 435-444.

Goldberg D. & Novoplansky (1997). On the relative importance of competition in unproductive environments. *Journal of Ecology* **85**, 409-418.

Goldberg D.E. & Barton D.E. (1992). Patterns and consequences of inter-specific competition in natural communities: A review of field experiments with plants. *American Naturalist* **139**, 771-801.

Goldberg D.E. & Landa K. (1991). Competitive effect and response – hierarchies and correlated traits in the early stages of competition. *Journal of Ecology* **79**, 1013-1030.

Goldberg D.E. & Werner P.A. (1983). Equivalence of competitors in plant communities: a null hypothesis and an experimental approach. *American Journal of Botany* **70**, 1098-1104.

Goldberg D.E., Rajaniemi T., Gurevitch J. & Stewart-Oaten A. (1999). Empirical approaches to quantifying interaction intensity: competition and facilitation along productivity gradients. *Ecology* **80**, 1118-1131.

Goldberg D.E., Turkington R. & Olsvig-Whittaker L. (1995). Quantifying the community-level consequences of competition. *Folia Geobotanica & Phytotaxonomica* **30**, 231-242.

Goldberg D.E., Turkington R., Olsvig-Whittaker L. & Dyer A.R. (2001). Density dependence in an annual plant community: Variation among life history stages. *Ecological Monographs* **71**, 423-446.

Goodacre R. (2005). Making sense of the metabolome using evolutionary computation: Seeing the wood with the trees. *Journal of Experimental Botany* **56**, 245-254.

Goodacre R. (2007). Metabolomics of a superorganism. *Journal of Nutrition* **137**, 259-266.

Goodacre R., Timmins É.M., Burton R., Kaderbhai N., Woodward A.W., Kell D.B. & Rooney P.J. (1998). Rapid identification of urinary tract infection bacteria using hyperspectral whole-organism fingerprinting and artificial neural networks. *Microbiology* **144**, 1157-1170.

Goodacre R., Timmins É.M., Rooney P.J., Rowland J.J. & Kell D.B. (1996). Rapid identification of *Streptococcus* and *Enterococcus* species using diffuse reflectance-absorbance Fourier transform infrared spectroscopy and artificial neural networks. *FEMS Microbiology Letters* **140**, 233-239.

Goodacre R., York E.V., Heald J. & Scott I.M. (2002). Chemometric discrimination of unfractionated plant extracts analyzed by electrospray mass spectroscopy. *Phytochemistry* **62**, 859-863.

Gosling P. (2005). Facilitation of *Urtica dioica* colonisation by *Lupinus arboreus* on a nutrient-poor mining soil. *Plant Ecology* **178**, 141-148.

Gough L., Goldberg D.E., Hershock C., Pauliukonis N. & Petru M. (2001). Investigating the community consequences of competition among clonal plants. *Evolutionary Ecology* **15**, 547-563.

Grace J.B. (1991). A clarification of the debate between Grime and Tilman. *Functional Ecology* **5**, 583-587.

Grace J.B. (1993). The effects of habitat productivity on competition intensity. *Trends in Ecology & Evolution* **8**, 229-230.

Grace J.B. (1995). On the measurement of plant competition intensity. *Ecology* **76**, 305-308.

Grace J.B., Keough J. & Guntenspergen G.R. (1992). Size bias in traditional analyses of substitutive competition experiments. *Oecologia* **90**, 429-434.

Green A.E.S. (1983). The penetration of ultraviolet-radiation to the ground. *Physiologia Plantarum* **58**, 351-359.

Grime J.P. (1974). Vegetation classification by reference to strategies. *Nature* **250**, 26-31.

Grime J.P. (1977). Evidence for the existence of 3 primary strategies in plants and its relevance to ecological and evolutionary theory. *American Naturalist* **111**, 1169-1194.

Grime J.P. (1979). *Plant Strategies & Vegetation Processes.* (John Wiley & Sons, Chichester).

Grime J.P. (1988). Critique of the triangular model of primary strategies – comment. *Ecology* **69**, 1618-1620.

Grime J.P. (1989). The stress debate – symptom of impending synthesis. *Biological Journal of the Linnaean Society* **37**, 3-17.

Grime J.P. (1997). Ecology – Biodiversity and ecosystem function: the debate deepens. *Science* **277**, 1260-1261.

Grime J.P., Hodgson J.G. & Hunt (1988). *Comparative Plant Ecology: A Functional Approach to Common British Species*. Unwin Hyman, London.

Groth A.T., Lovett-Doust L. & Lovett-Doust J. (1996). Population density and module demography in *Trapa natans* (Trapaceae), an annual, clonal aquatic macrophyte. *American Journal of Botany* **83**, 1406-1415.

Grubb P.J., Ford M.A. & Rochefort L. (1997). The control of relative abundance of perennials in chalk grassland: Is root competition or shoot competition more important? *Phytocoenologia* **27**, 289-309.

Gwynn Jones D., Johansson U., Phoenix G.K., Gehrke C., Callaghan T.V., Björn L.O., Sonesson M. & Lee J.A. (1999a). UV-B impacts and interactions with other co-occurring variables of environmental change: An Arctic perspective. In: Stratospheric Ozone Depletion. The effects of enhanced UV-B radiation on terrestrial ecosystems (Ed. J. Rozema), pp 187-201. Backhuys Publishers, Leiden.

Gwynn Jones D., Lee J.A. & Callaghan T.V. (1997). Effects of enhanced UV-B radiation and elevated CO_2 concentrations on a sub-arctic forest heath ecosystem. *Plant Ecology* **128**, 242-249.

Gwynn Jones D., Lee J.A., Johansson U., Phoenix G.K., Callaghan T.V. & Sonesson M. (1999b). The responses of plant functional types to enhanced UV-B radiation. In:

Stratospheric Ozone Depletion. The effects of enhanced UV-B radiation on terrestrial ecosystems (Ed. J. Rozema), pp 173-185. Backhuys Publishers, Leiden.

Gwynn-Jones D., Huang W., Easton G., Goodacre R. & Scullion J. (2004). UV-B radiation induced changes in litter quality affects earthworm growth and cast characteristics as determined by metabolic fingerprinting. *Pedobiologia* **47**, 784-787.

Hamed S. & Dignan J. (1992). Global emissions of nitrogen and sulfur oxides in fossil fuel combustion 1970-1986. *Journal of the Air and Waste Management Association* **42**, 159-163.

Hansson M.L. & Göransson A. (1993). Growth and partitioning of *Anthriscus sylvestris* (L.) Hoffm. And *Festuca ovina* (L.) at different relative addition rates of nitrogen. *Plant & Soil* **156**, 187-190.

Harrigan G.G., La Plante R.H., Cosma G.N., Cockerell G., Goodacre R., Maddox J.F., Luyendyk J.P., Ganey P.E. & Roth R.A. (2004). Application of high-throughput Fourier-Transform infrared spectroscopy in toxicology studies: Contributions to a study on the development of an animal model for idosyncratic toxicology. *Toxicology Letters* **146**, 197-205.

Harte J. & Shaw R. (1995). Shifting dominance within a montane vegetation community – results of a climate warming experiment. *Science* **267**, 876-880.

Hartley S.E. & Amos L. (1999). Competitive interactions between *Nardus stricta* L. and *Calluna vulgaris* (L.) Hull: The effect of fertilizer and defoliation on above- and below-ground performance. *Journal of Ecology* **87**, 330-340.

Hasegawa P.M. & Bressan R.A. (2000). Plant cellular & molecular responses to high salinity. *Annual Review of Plant Physiology & Plant Molecular Biology* **51**, 463-499.

Hastwell G.T. & Panetta F.D. (2005). Can differential responses to nutrients explain the success of environmental weeds? *Journal of Vegetation Science* **16**, 77-84.

Hauggaard-Nielsen H. & Jensen E.S. (2005). Facilitative root interactions in intercrops. *Plant & Soil* **274**, 237-250.

Havström M., Callaghan T.V. & Jonasson J. (1993). Differential growth-responses of *Cassiope tetragona*, an arctic dwarf-shrub, to environmental perturbations among three contrasting high sites and sub-arctic sites. *Oikos* **66**, 389-402.

Heijden M.G.A., Bakker R., Verwaal J., Scheublin T.R., Rutten M., van Logtestijn R. & Staehlin C. (2006). Symbiotic bacteria as a determinant of plant community structure and plant productivity in dune grassland. *FEMS Microbiology Ecology* **56**, 178-187.

Helm D., Labichinski H., Schallehn G. & Naumann D. (1991). Classification and identification of bacteria by Fourier-transform infrared spectroscopy. *Journal of General Microbiology* **137**, 69-79.

Høgh-Jensen H. & Schjoerring J.K. (2000). Below-ground nitrogen transfer between different grassland species: Direct quantification by N-15 leaf feeding compared with indirect dilution of soil N-15. *Plant & Soil* **227**, 171-183.

Høgh-Jensen H. (2006). The nitrogen transfer between plants: An important but difficult flux to quantify. *Plant & Soil* **282**, 1-5.

Holland E.A., Braswell B.H., Sulzman J. & Lamarque J.F. (1995). Nitrogen deposition onto the US and Western Europe: Synthesis of observations and models. *Ecological Applications* **15**, 38-57.

Holmes M.G. (2002). An outdoor multiple wavelength system for the irradiation of biological samples: analysis of the long-term performance of various lamps and filter combinations. *Photochemistry and Photobiology* **76**, 158-163.

Hooper D.U. & Dukes J.S. (2004). Overyielding among plant functional groups in a long-term experiment. *Ecology Letters* **7**, 95-105.

Huang W.E., Hopper W.E., Goodacre R., Beckmann M. & Draper J. (2006). Rapid characterization of microbial biodegradation pathways by FT-IR spectroscopy. *Journal of Microbiological Methods* **67**, 273-280.

Huiskes A.H.L., Lud D., Moerdijk-Poortvliet T.C.W. & Rozema J. (1999). Impact of UV-B radiation on Antarctic terrestrial vegetation. In: Stratospheric Ozone Depletion. The effects of enhanced UV-B radiation on terrestrial ecosystems (Ed. J. Rozema), pp 313-337. Backhuys Publishers, Leiden.

Hulme P.D., Pakeman R.J., Torvell L., Fisher J.M. & Gordon I.J. (1999). The effects of controlled sheep grazing on the dynamics of upland *Agrostis-Festuca* grassland. *Journal of Applied Ecology* **36**, 886-900.

Hutchings M.J. & Budd C.S.J. (1981). Plant competition and its course through time. *Bioscience* **31**, 640-645.

Inouyé R.S. & Schaffer W.M. (1981). On the ecological meaning of ratio (de Wit) diagrams in plant ecology. *Ecology* **62**, 1679-1681.

Isacch J.P., Costa C.S.B., Rodriguez-Gallego L., Conde D., Escapada M., Gagliardini D.A. & Iribane O.O. (2006). Distribution of saltmarsh plant communities associated with environmental factors along a latitudinal gradient on the south-west Atlantic coast. *Journal of Biogeography* **33**, 888-900.

Ito M., Miyamoto J., Mori Y., Fujimoto S., Uchiumi T., Abe M., Suzuki A., Tabata S. & Fukui K. (2004). Genome and chromosome dimensions of *Lotus japonicus*. *Journal of Plant Research* **113**, 435-442.

Jarvis R.M. & Goodacre R. (2004). Genetic algorithm optimization for pre-processing and variable selection of spetroscopic data. *Bioinformatics* **21**, 860-868.

Jeffrey D.W. & Pigott C.D. (1973). The response of grasslands on sugar-limestone in Teesdale to application of phosphorus and nitrogen. *Journal of Ecology* **61**, 85-92.

Jenkins H., Hardy N., Beckmann M., Draper J., Smith A.R., Taylor J., Fiehn O., Goodacre R., Bino R.J., Hall R., Kopka J., Lane G.A., Lange B.M., Liu J.R., Mendes P., Nikolau B.J., Oliver S.G., Paton N.W., Rhee S., Roessner-Tunali U., Saito K., Smedsgaard J., Sumner L.W., Wang T., Walsh S., Wurtele E.S. & Kell D.B. (2004). A proposed framework for the description of plant metabolomics experiments and their results. *Nature Biotechnology* **22**, 1601-1606.

Johanson U., Gehrke C., Björn L.O. & Callaghan T.V. (1995a). The effects of enhanced UV-B radiation on the growth of dwarf shrubs in a sub-arctic heathland. *Functional Ecology* **9**, 713-719.

Johanson U., Gehrke C., Björn L.O., Callaghan T.V. & Sonesson M. (1995b). The effects of enhanced UV-B radiation on a sub-Arctic heath ecosystem. *Ambio* **24**, 106-111.

Johanson U., Kyparissis A., Levizou E., Nikopoulos D., Petropoulou Y. & Stephanou M. (1997). The effects of UV-B radiation on European heathland species. *Plant Ecology* **128**, 252-264.

Johnson D. (2003). Response of terrestrial microorganisms to ultraviolet-B radiation in ecosystems. *Research in Microbiology* **154**, 315-320.

Johnson D., Campbell C.D., Lee J.A., Callaghan T.V. & Gwynn Jones D. (2002). Arctic microorganisms respond more to elevated UV-B radiation than CO_2. *Nature* **416**, 82-83.

Johnson H.E., Broadhurst D., Goodacre R. & Smith A.R. (2003). Metabolomic fingerprinting of salt-stressed tomatoes. *Phytochemistry* **62**, 919-928.

Johnson H.E., Broadhurst D., Kell D.B., Theodorou M.K., Merry R.J. & Griffith G.W. (2004). High-throughput metabolic fingerprinting of legume silage fermentations via Fourier Transform infrared spectroscopy and chemometrics. *Applied and Environmental Microbiology* **70**, 1583-1592.

Johnson H.E., Gilbert R.J., Winson M.K., Goodacre R., Smith A.R., Rowland J.J., Hall M.A. & Kell D.B. (2000). Explanatory analysis of the metabolome using genetic programming of simple, interpretable rules. *Genetic Programming and Evolvable Machines* **1**, 243-258.

Joliffe P.A., Minjas A.N. & Runeckles V.C. (1984). A reinterpretation of yield relationships in replacement series experiments. *Journal of Applied Ecology* **21**, 227-243.

Jordan B.R. (2002). Molecular responses of plant cells to UV-B stress. *Functional Plant Biology* **29**, 909-916.

Jordan T.E. & Weller D.E. (1996). Human contributions to terrestrial nitrogen flux. *Bioscience* **46**, 655-664.

Kaderbhai N.N., Broadhurst D.I., Ellis D.I., Goodacre R. & Kell D.B. (2003). Functional genomics via metabolic fingerprinting: Monitoring metabolite secretion by *Escerichia coli* tryptophan metabolism using FT-IR and direct injection electrospray mass spectrometry. *Comparative & Functional Genomics* **4**, 376-391.

Kadiver H. & Stapleton A.E. (2004). Ultraviolet radiation alters maize phyllosphere bacterial diversity. *Microbial Ecology* **45**, 353-361.

Keddy P., Gaudet C. & Fraser L.H. (2000). Effects of high and low nutrients on the competitive hierarchy of 26 shoreline plants. *Journal of Ecology* **83**, 413-423.

Keddy P., Nielsen K., Weiher E. & Lawson R. (2002). Relative competitive performance of 63 species of terrestrial herbaceous plants. *Journal of Vegetation Science* **13**, 5-16.

Keddy P., Twolan-Strutt L. & Shipley B. (1997). Experimental evidence that interspecific competitive asymmetry increases with soil productivity. *Oikos* **80**, 253-256.

Keddy P.A., Twolan-Strutt L. & Wisheu I.C. (1994). Competitive effect and response rankings in 20 wetland plants – are they consistent across 3 environments. *Journal of Ecology* **82**, 635-643.

Keiller D.R. & Holmes M.G. (2001). Effects of long-term exposure to elevated UV-B radiation on the photosynthetic performance of five broad-leaved tree species. *Photosynthesis Research* **67**, 229-240.

Kenney B. & Schockor J.P. (2003). Complementary NMR & LC-MS technologies for metabonomic studies. *Pharmagenomics* **1**, 56-63.

Kerr R.A. (1988). Evidence of Arctic ozone destruction. *Science* **240**, 1144-1145.

Klironomos J.N. & Allen M.F. (1995). UV-B-mediated changes on belowground communities associated with the roots of *Acer saccharum*. *Functional Ecology* **9**, 923-930.

Kreuzaler F., Ragg H., Fautz E., Kuhn D.N. & Hahlbrock K. (1983). UV-Induction of chalcone synthase mRNA in cell suspension cultures of *Petroselenium hortense*. *Proceedings of the National Academy of Sciences, USA* **80**, 2591-2593.

Krizek D.T. & Mirecki R.M. (2004). Evidence for phytotoxic effects of cellulose acetate in UV exclusion studies. *Environmental & Experimental Botany* **51**, 33-43.

Krizek D.T. (2004). Influence of PAR and UV-A in determining plant-sensitivity and photomorphogenic responses to UV-B radiation. *Photochemistry & Photobiology* **79**, 307-315.

Krupa S.V. (2003). Effects of atmospheric ammonia (NH_3) on terrestrial vegetation: a review. *Environmental Pollution* **124**, 179-221.

Kuijper D.P.J., Dubbeld J. & Bakker J.P. (2005). Competition between two grass species with and without grazing over a productivity gradient. *Plant Ecology* **179**, 237-246.

La Peyre M.K.G., Grace J.B., Hahn E. & Mendelssohn I.A. (2001). The importance of competition in regulating plant species abundance along a salinity gradient. *Ecology* **82**, 62-69.

Lambers J.H.R., Harpole W.S., Tilman D., Knops J. & Reich P.B. (2004). Mechanisms responsible for the positive diversity-productivity relationship in Minnesota grasslands. *Ecology* **7**, 661-668.

Lanta V. & Leps J. (2006). Effect of functional group richness and species richness in manipulated productivity-diversity studies: A glasshouse pot experiment. *Acta Oecologia – International Journal of Ecology* **29**, 85-96.

Law R. & Watkinson A.R. (1987). Response-surface analysis of two-species competition: An experiment on *Phleum arenarium* and *Vulpia fasciculata*. *Journal of Ecology* **75**, 871-886.

Lee J.A. (1998). Unintentional experiments in terrestrial ecosystems: Ecological effects of sulphur and nitrogen pollutants. *Journal of Ecology* **86**, 1-12.

Lee J.A., Caporn S.J.M. & Read D.J. (1992). Effect of increasing nitrogen deposition and acidification on heathlands. Acidification Research, Evaluation & Policy Applications (ed. T. Schneider) pp. 97-106, Elsevier, Amsterdam.

Lenssen J.P.M. & De Kroon H. (2005). Abiotic constraints at the upper boundaries of two *Rumex* species on a freshwater flooding gradient. *Journal of Ecology* **93**, 138-147.

Levizou E. & Manetas Y. (2001). Combined effects of enhanced UV-B radiation and additional nutrients on growth of two Mediterranean plant species. *Plant Ecology* **154**, 179-186.

Li L., Zhang F.S., Li X.L., Christie P., Sun J.H., Yang S.C. & Tang C.X. (2003). Inter-specific facilitation of nutrient uptake by intercropped maize and faba bean. *Nutrient Cycling in Agroecosystems* **65**, 61-71.

Liakoura V., Stefanou M., Manetas Y., Cholevas C. & Karabourniotis G. (1997). Trichome density and its UV-B protective potential are affected by shading and leaf position on the canopy. *Environmental and Experimental Botany* **38**, 223-229.

Lodge G.M. (2002). Effects of continuous grazing and seasonal closures on the performance and persistence of some sown temperate perennial grasses, North-West Slopes New South Wales. *Australian Journal of Experimental Agriculture* **42**, 431-438.

Loehle C. (1988). Problems with the triangular model for representing plant strategies. *Ecology* **69**, 284-286.

Luscher A., Connolly J. & Jacquard P. (1992). Neighbour specificity between *Lolium perenne* and *Trifolium repens* from a natural pasture. *Oecologia* **91**, 404-409.

Mackerness S.A.H. (2000). Plant responses to ultraviolet-B (UV-B: 280-320 nm) stress: What are the key regulators? Invited review. *Plant Growth Regulation* **32**, 27-39.

Manetas Y. (1999). Is enhanced UV-B radiation really damaging for plants? Some case studies with European Mediterranean species. In: Stratospheric Ozone Depletion. The effects of enhanced UV-B radiation on terrestrial ecosystems (Ed. J. Rozema), pp 251-263. Backhuys Publishers, Leiden.

Márquez A.J., Betti M., García-Calderón M., Pal'ove-Balang P., Díaz P. & Monza J. (2005). Nitrate assimilation in *Lotus japonicus*. *Journal of Experimental Botany* **56**, 1741-1749.

Marquez V.J. & Allen E.B. (1996). Ineffectiveness of two annual legumes as nurse plants for establishment of *Artemisia californica* in coastal sage scrub. *Restoration Ecology* **4**, 42-50.

Matthews E. (1994). Nitrogenous fertilizers – global distribution of consumption and associated emissions of nitrous-oxides and ammonia. *Global Biogeochemical Cycles* **8**, 411-439.

Mattioli L., Cangi F., Maidecchi A., Ghiara C., Ragazzi E., Tubaro M., Stella L., Tisato F. & Traldi P. (2006). Metabolic fingerprinting of plant extracts. *Journal of Mass Spectrometry* **41**, 1534-1545.

McElroy M.B., Salawith R.J., Wofsy S.C. & Logan J.A. (1986). Reductions of Antarctic ozone due to synthesis interactions of chlorine and bromine. *Nature* **321**, 759-762.

McKenzie R.L., Björn L.O., Bais A. & Ilyas M. (2003). Changes in biologically active radiation reaching the Earth's surface. In: *Environmental effects of ozone depletion and its interactions with climate change (UNEP).*

McKenzie R.L., Paulin K.J. & Madronich S. (1998). Effects of show cover on UV irradiance and surface albedo: A case study. *Journal of Geophysical Research – Atmospheres* **103**, 28785-28792.

McLeod A.R. (1997). Outdoor supplementation systems for studies of the effects of increased UV-B radiation. *Plant Ecology* **128**, 78-92.

Menchaca L. & Connolly J. (1990). Species interference in Clover-Ryegrass mixtures. *Journal of Ecology* **78**, 223-232.

Mendham D.S., Kumaraswamy S., Balasundaran M., Sankaran K.V., Corbeels M., Grove T.S., O'Connell A.M. & Rance S.J. (2004). Legume cover cropping effects on early growth and soil nitrogen supply in eucalypt plantations in south-western India. *Biology & Fertility of Soils* **39**, 375-382.

Mepstead R., Paul N.D., Stephen J., Cowlett J.E., Nogues S., Baker N.R., Jones H.G. & Agnes P.G. (1996). Effects of enhanced UV-B radiation on Pea (*Pisum sativum* L.) grown under field conditions in the U.K.. *Global Change Biology* **2**, 325-334.

Michelsen A., Graglia E., Schmidt I.K., Jonasson S., Sleep D. & Quarmby C. (1999). Differential responses of grass and a dwarf shrub to long-term changes in soil microbial biomass C, N and P following factorial addition of NPK fertilizer, fungicide and labile carbon to a heath. *New Phytologist* **143**, 523-538.

Middleton E.M. & Teramura A.H. (1994). Understanding photosynthesis, pigment & growth response induced by UV-B and UV-A irradiances. *Photochemistry and Photobiology* **60**, 38-45.

Milne J.A., Pakeman R.J., Kirkham F.W., Jones I.P & Hossell J.E. (2002). Biomass production of upland vegetation types in England and Wales. *Grass & Forage Science* **57**, 373-388.

Molina M.J. & Rowland F.S. (1974). Stratospheric sink for chlorofluoromethanes: Chlorine atom-catalysed destruction of ozone. *Nature* **249**, 810-812.

Molina M.J., Tso T.-L., Molina L.T. & Wong C.-Y. (1987). Antarctic stratospheric chemistry of chlorine nitrate, hydrogen cyanide and ice: Release of active chlorine. *Science* **238**, 1253-1257.

Molina M.J., Zhong R., Wooldridge P.J., McMahon J.R., Kim J.E., Chong H.Y. & Beyer K.D. (1993). Physical chemistry of the $H_2SO_4/HNO_3/H_2O$ system: Implications for polar stratospheric clouds. *Science* **261**, 1418-1423.

Morecroft M.D., Sellers E.K. & Lee J.A. (1994). An experimental investigation into the effects of atmospheric nitrogen deposition on two semi-natural grasslands. *Journal of Ecology* **82**, 475-483.

Mount G.H., Soloman S., Sandes R.W., Jakoubek R.O. & Schmeltehopf A.L. (1988). Observations of stratospheric NO_2 & O_3 at Thule, Greenland. *Science* **242**, 555-558.

Moyer-Henry K.A., Burton J.W., Israel D. & Rufty T. (2006). Nitrogen transfer between plants: A N-15 natural abundance study with crop and weed species. *Plant & Soil* **282**, 7-20.

Müller R., Crutzen P.J., Grooss J.-U., Brühl C., Rusell III J.M., Gernandt H., McKenna D.S. & Truck A.F. (1997). Severe chemical ozone losses in the Arctic during the winter of 1995-96. *Nature* **389**, 709-712.

Musil C.F. & Wand S.J.E. (1999). Impact of UV-B radiation on South African mediterranean ecosystems. In: Stratospheric Ozone Depletion. The effects of

enhanced UV-B radiation on terrestrial ecosystems (Ed. J. Rozema), pp 265-291. Backhuys Publishers, Leiden.

Musil C.F., Björn L.O., Scourfield M.W.J. & Bodeker G.E. (2002). How substantial are ultraviolet-B supplementation inaccuracies in experimental square-wave delivery systems? *Environmental & Experimental Botany* **47**, 25-38.

Musil C.F., Kgope B.S., Chimphango S.B.M. & Dakora F.D. (2003). Nitrate additions enhance the photosynthetic sensitivity of a nodulated South African Mediterranean-climate legume (*Podalyria calyptrata*) to elevated UV-B. *Environmental & Experimental Botany* **50**, 197-210.

Nadelhoffer K.J. (2000). The potential effects of nitrogen deposition on fine-root production in forest ecosystems. *New Phytologist* **147**, 131-139.

Nagel L.M., Bassman J.H., Edwards G.E., Robberecht R. & Franceshi V.R. (1998). Leaf anatomical changes in *Populus trichocarpa*, *Quercus rubra*, *Pseudotsuga menziesii* and *Pinus ponderosa* exposed to enhanced ultraviolet-B radiation. *Physiologia Plantarum* **104**, 385-396.

Newsham K.K., Greenslade P.D. & McLeod A.R. (1999). Effects of elevated ultraviolet radiation on *Quercus robur* and its insect and ectomycorrhizal associates. *Global Change Biology* **5**, 881-890.

Newsham K.K., McLeod A.R., Greenslade P.D. & Emmett B.A. (1996). Appropriate controls in outdoor UV-B supplementation experiments. *Global Change Biology* **2**, 319-324.

Niang A., Styger E., Gahamanyi A., Hoekstra D. & Coe R. (1998). Fodder-quality improvement through contour planting of legume-shrub/grass mixtures in croplands of Rwanda highlands. *Agroforestry Systems* **39**, 263-274.

Niemi R., Martikainen P.J., Silvola J., Wulff A., Turtola S. & Holopainen T. (2002). Elevated UV-B radiation alters fluxes of methane and carbon dioxide in peatland microcosms. *Global Change Biology* **8**, 361-371.

Nolan T., Connolly J. & Wachendorf M. (2001). Mixed grazing and climatic determinants of white clover (*Trifolium repens* L.) content in a permanent pasture. *Annals of Botany* **88**, 713-724.

Norby R.J. (1995). Nitrogen deposition: A component of global climate change analyses. *New Phytologist* **139**, 189-200.

Norton L.R., McLeod A.R., Greenslade P.D., Firbank L.G. & Watkinson A.R. (1999). Elevated UV-B radiation effects on experimental grassland communities. *Global Change Biology* **5**, 601-608.

Oliver S.G., Winson M.K., Kell D.B. & Baganz F. (1998). Systematic functional analysis of the yeast genome. *Trends in Biotechnology* **16**, 373-378.

Pacala S.W. & Tilman D. (1994). Limiting similarity in mechanistic and spatial models of plant competition in heterogeneous environments. *American Naturalist* **143**, 222-257.

Pan X.Y., Geng Y.P., Zhang W.J., Li B. & Chen J.K. (2006). The influence of abiotic stress and phenotypic plasticity on the distribution of invasive *Alternanthera philoxeroides* along a riparian zone. *Acta Oecologia – International Journal of Ecology* **30**, 333-341.

Parsons A.N., Press M.C., Wookey P.A., Welker J.M., Robinson C.H., Callaghan T.V. & Lee J.A. (1995). Growth-responses of *Calamagrostis lapponica* to simulated environmental change in the sub-Arctic. *Oikos* **72**, 61-66.

Paul N.D. & Gwynn Jones D. (2003). Ecological roles of solar UV radiation: Towards an integrated approach. *Trends in Ecology & Evolution* **18**, 48-55.

Paul N.D. (2001). Plant responses to UV-B: Time to look beyond stratospheric ozone depletion? *New Phytologist* **150**, 5-8.

Peltzer D.A., Wilson S.D. & Gerry A.K. (1998). Competition along a productivity gradient in a low-diversity grassland. *American Naturalist* **151**, 465-476.

Pennings S.C., Grant M.B. & Bertness M.D. (2005). Plant zonation in low-latitude salt marshes: Disentangling the roles of flooding, salinity and competition. *Journal of Ecology* **93**, 159-167.

Petropoulou Y., Kyparissis A., Nikolopoulos D. & Manetas Y. (1995). Enhanced UV-B radiation alleviates the adverse-effects of summer drought in 2 Mediterranean pines under field conditions. *Physiologia Plantarum* **94**, 37-44.

Phoenix G.K., Gwynn Jones D., Callaghan T.V., Sleep D. & Lee J.D. (2001). Effects of global change on a sub-arctic heath: Effects of enhanced UV-B radiation and increased summer precipitation. *Journal of Ecology* **89**, 256-267.

Phoenix G.K., Gwynn Jones D., Lee J.A. & Callaghan T.V. (2000). The impacts of UV-B radiation on the regeneration of a sub-arctic heath community. *Plant Ecology* **146**, 67-75.

Pinto M.E., Edwards G.E., Riquelme A.A. & Ku M.S.B. (2002). Enhancement of nodulation in bean (*Phaseolus vulgaris*) by UV-B radiation. *Functional Plant Biology* **29**, 1189-1196.

Powers S.A., Ashmore M.R., Cousins D.A. & Sheppard L.J. (1998). Effects of nitrogen addition on the stress sensitivity of *Calluna vulgaris*. *New Phytologist* **138**, 663-673.

Prospero J.M., Barrett K., Church T., Dentener F., Duce R.A., Galloway J.N., Levy H., Moody J., Quinn P. (1996). Atmospheric deposition of nutrients to the North Atlantic Basin. Biogeochemistry 35, 27-73.

Putnam D.H. & Allan D.L. (1992). Mechanism for overyielding in a sunflower mustard intercrop. *Agronomy Journal* **84**, 188-195.

Quaite F.E., Sutherland B.M. & Sutherland J.C. (1992). Action spectrum for DNA damage in alfalfa lowers predicted impact of ozone depletion. *Nature* **358**, 576-578.

Rajaniemi T.K. & Goldberg D.E. (2000). Quantifying individual- and community-level effects of competition using experimentally-determined null species pools. *Journal of Vegetation Science* **11**, 433-442.

Ramseier D., Connolly J. & Bazzaz F.A. (2005). Carbon dioxide regime, species identity and influence of species initial abundance as determinants of change in stand biomass composition in five-species communities: An investigation using a simplex design and RGRD analysis. *Journal of Ecology* **93**, 502-511.

Rasmussen J., Eriksen J., Jensen E.S., Esbensen K.H. & Høgh-Jensen H. (2007). In situ carbon and nitrogen dynamics in ryegrass-clover mixtures: Transfers, deposition and leaching. *Soil Biology & Biochemistry* **39**, 804-815.

Ratcliffe D.A. & Thompson D.B.A. (1988). The British uplands: Their ecological character and international significance. In: Usher MB, Thompson DBA, Eds: Ecological change in the uplands. Oxford, UK: Blackwell Scientific Publications, 9–36.

Redmond J.W., Batley M., Djordjevic M.A., Innes R.W., Kuempel P.L. & Rolfe B.G. (1986). Flavones induce expression of nodulation genes in *Rhizobium*. *Nature* **323**, 632-635.

Rex M., Harris N.R.P., von der Gathen P., Lehmann R., Braathen G.O., Reimer E., Beck A., Chipperfield M.P., Alfier R., Allaart M., O'Connor F., Dier H., Dorokhov V., Fast H., Gil M., Kyro E., Litynska Z., Mikkelsen I.S., Molyneux M.G., Nakane H., Notholt J., Rummukainen M., Viatte P. & Wenger J. (1997). Prolonged stratospheric ozone loss in the 1995-1996 Arctic winter. *Nature* **389**, 835-838.

Rinnan R., Gehrke C. & Michelsen A. (2006). Two mire species respond differently to enhanced ultraviolet-B radiation: Effects on biomass allocation and root exudation. *New Phytologist* **169**, 809-818.

Rinnan R., Keinanen M.M., Kasurinen A., Asikainen J., Kekki T.K., Holopainen T., Ro-Poulsen H., Mikkelsen T.N. & Michelsen A. (2005). Ambient ultraviolet radiation in the Arctic reduces root biomass and alters microbial community composition but has no effects on microbial biomass. *Global Change Biology* **11**, 564-574.

Rinnan R., Michelsen A., Baath E. & Jonasson S. (2007). Fifteen years of climate change manipulations alter soil microbial communities in a subarctic heath ecosystem. *Global Change Biology* **13**, 28-39.

Roberts M.R. & Paul N.D. (2006). Seduced by the dark side: Integrating molecular and ecological perspectives on the influence of light and plant defence against pests and pathogens. *New Phytologist* **170**, 677-699.

Robinson C.H., Wookey P.A., Lee J.A., Callaghan T.V. & Press M. (1998). Plant community responses to simulated environmental change at a high arctic polar semi-desert, Svalbard (79°N). *Ecology* **79**, 856-866.

Robson T.M., Pancotto V.A., Flint S.D., Ballaré C.L., Salo O.E., Scopel A.L. & Caldwell M.M. (2003). Six years of solar UV-B manipulations affect growth of sphagnum and vascular plants in a Tierra del Fuego peatland. *New Phytologist* **160**, 379-389.

Rodriguez-Echeverria S. & Perez-Fernandez M.A. (2003). Soil fertility and herb facilitation mediated by *Retama sphaerocarpa*. *Journal of Vegetation Science* **14**, 807-814.

Rodwell J.S. ed. (1993). *British Plant Communities. Volume 4. Grasslands and montane communities*. Cambridge, Cambridge University Press.

Rosch H., Van Rooyen M.W. & Theron G.K. (1997). Competitive effect and response of ten Namaqualand ephemeral plant species at two nutrient levels. *South African Journal of Botany* **63**, 210-215.

Rousseaux M.C., Ballaré C.L., Scopel A.L., Searles P.S. & Caldwell M.M. (1998). Solar UV-B radiation affects plant-insect interactions in a natural ecosystem of Tierra del Fuego (South Argentina). *Oecologia* **116**, 528-535.

Rousseaux M.C., Scopel A.L., Searles P.S., Caldwell M.M., Salo O.E. & Ballaré C.L. (2001). Response to solar ultraviolet-B radiation in a shrub-dominated natural ecosystem of Tierra del Fuego (South Argentina). *Global Change Biology* **7**, 467-478.

Rozema J. (1999). UV-B radiation and terrestrial ecosystems: Processes, structure and feedback loops. In: Stratospheric Ozone Depletion. The effects of enhanced UV-B radiation on terrestrial ecosystems (Ed. J. Rozema), pp 101-115. Backhuys Publishers, Leiden.

Rozema J., Boelen P., Solheim B., Zielke H., Buskens A., Doorenbosch M., Fijn R., Herder J., Callaghan T., Björn L.O., Gwynn Jones D., Broekman R., Blokker P. & van de Poll W. (2006). Stratospheric ozone depletion: High Arctic tundra plant growth on Svalbard is not affected by enhanced UV-B after seven years of UV-B supplementation in the field. *Plant Ecology* **182**, 121-135.

Rozema J., Lenssen G.M., Van de Staaij J.W.M., Tosserams M., Visser A.J. & Broekman R.A. (1997a). Effects of UV-B radiation on terrestrial plants and ecosystems: Interactions with CO_2 enrichment. *Plant Ecology* **128**, 182-191.

Rozema J., van der Staaij J., Björn L.O. & Caldwell M. (1997b). UV-B as an environmental factor in plant life: Stress and regulation. *Trends in Ecology & Evolution* **12**, 22-28.

Rustad L.E., Campbell J.L., Marion G.M., Norby R.J., Mitchell M.J., Hartley A.E., Cornelissen J.H.C. & Gurevitch J. (2001). A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. *Oecologia* **126**, 543-562.

Ryan D. & Robards K. (2006). Metabolomics: The greatest omics of them all? *Analytical Chemistry* **78**, 7954-7958.

Rydin H. & Bengtsson J. (1990). Competition theory: Towards a synthesis? *Journal of Vegetation Science* **1**, 567-569.

Sampson B.J. & Cane J.H. (1999). Impact of enhanced ultraviolet-B radiation on flower, pollen and nectar production. *American Journal of Botany* **86**, 108-114.

Schauer N. & Fernie A.R. (2006). Plant metabolomics: Towards biological function and mechanism. *Trends in Plant Science* **11**, 508-516.

Scotto J., Cotton G., Urbach F., Berger D. & Fears T. (1988). Biologically effective ultraviolet-radiation – surface measurements in the United-States, 1974 to 1985. *Science* **239**, 762-764.

Scullion J., Elliott G.N., Huang W.E., Goodacre R., Worgan H., Darby R., Bailey M.J., Gwynn-Jones D., Griffith G.W., Winson M.K., Williams P.A., Clegg C. & Draper J. (2003). Use of earthworm casts to validate FT-IR spectroscopy as a 'sentinel' technology for high-throughput monitoring of global changes in microbial ecology. *Pedobiologia* **47**, 440-446.

Searles P.S., Flint S.D., Díaz S.B., Rousseaux M.C., Ballaré C.L. & Caldwell M.M. (1999). Solar ultraviolet-B radiation influence on *Sphagnum* bog and *Carex* fen ecosystems: First year season findings in Tierra del Fuego, Argentina. *Global Change Biology* **5**, 225-234.

Searles P.S., Flint S.D., Díaz S.B., Rousseaux M.C., Ballaré C.L. & Caldwell M.M. (2002). Plant response to solar ultraviolet-B radiation in a southern American *Sphagnum* peatland. *Journal of Ecology* **90**, 704-713.

Searles P.S., Kropp B.R., Flint S.D. & Caldwell M.M. (2001). Influence of solar UV-B radiation on peatland microbial communities of south Argentina. *New Phytologist* **152**, 213-221.

Sebastia M.T. (2007). Plant guilds drive biomass response to global warming and water availability in subalpine grassland. *Journal of Applied Ecology* **44**, 158-167.

Seger C. & Sturm S. (2007). Analytical aspects of plant metabolite profiling patterns: Current standings and future aims. *Journal of Proteome Research* **6**, 480-497.

Shilo-Volin H., Novoplansky A., Goldberg D.E. & Turkington R. (2005). Density regulation in annual plant communities under variable resource levels. *Oikos* **108**, 241-252.

Shinozaki K. & Yamaguchi-Shinozaki K. (2000). Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signalling pathways. *Current Opinion Plant Biology* **3**, 217-223.

Shiozaki N., Hattori I., Gojo R. & Tezuka T. (1999). Activation of growth and nodulation in a symbiotic system between pea plants and leguminous bacteria by near-UV radiation. *Journal of Photochemistry & Photobiology B – Biology* **50**, 33-37.

Shirley B.W. (1996). Flavonoid biosysnthesis: 'New' functions for an 'Old' pathway. *Trends in Plant Science* **1**, 377-382.

Silvertown J. & Lovett-Doust (1993). *Introduction to plant population biology.* (3rd ed., Blackwell Scientific, Oxford).

Singh A. (1996). Growth, physiological, and biochemical responses of three tropical legumes to enhanced UV-B radiation. *Canadian Journal of Botany – Révue Canadienne de Botanique* **74**, 135-139.

Singh A. (1997). Increased UV-B radiation reduces N_2-fixation in tropical leguminous crops. *Environmental Pollution* **95**, 289-291.

Siqueira J.O., Safir G.R. & Nair M.G. (1991). Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. *New Phytologist* **118**, 87-93.

Skelton L.E. & Barrett G.W. (2005). A comparison of conventional and alternative agroecosystems using alfalfa (*Medicago sativa*) and winter wheat (*Triticum aestivum*). *Renewable Agriculture & Food Systems* **20**, 38-47.

Smith C.T., Elston J. & Bunting A.H. (1971). The effects of cutting and fertilizer treatment on the yield and botanical composition of chalk turfs. *Journal of the British Grassland Society* **26**, 213-219.

Smith R.S., Buckingham H., Bullard M.J., Shiel R.S. & Younger A. (1996). The conservation management of mesotrophic (meadow) grassland in northern England .1. Effects of grazing, cutting date and fertilizer on the vegetation of a traditionally managed sward. *Grass & Forage Science* **51**, 278-291.

Smith T. & Huston M. (2004). A theory of the spatial and temporal dynamics of plant communities. *Plant Ecology* **83**, 49-69.

Snaydon R.W. (1991). Replacement or additive designs for competition studies? *Journal of Applied Ecology* **31**, 784-786.

Solheim B., Johanson U., Callaghan T.V., Lee J.A., Gwynn Jones D. & Björn L.O. (2002). The nitrogen fixation potential of arctic cryptogram [sic.] species is influenced by enhanced UV-B radiation. *Oecologia* **133**, 90-93.

Soussana J.F. & Lafarge (1998). Competition for resources between neighbouring species and patch scale vegetation dynamics in temperate grasslands. *Annales de Zootechnie* **47**, 371-382.

Springer T.L., Aiken G.E. & McNew R.W. (2001). Combining ability of binary mixtures of native, warm-season grasses and legumes. *Crop Science* **41**, 818-823.

Stevens C.J., Dise N.B., Gowing D.J.G. & Mountford J.O. (2006). Loss of forb diversity in relation to nitrogen deposition in the UK: Regional trends and potential controls. *Global Change Biology* **12**, 1823-1833.

Stolarski R.S., Krueger A.J., Shoeber M.R., McPeters R.D., Newman P.A. & Alpert J.C. (1986). Nimbus 7 satellite measurements of the springtime Antarctic ozone decrease. *Nature* **322**, 808-811.

Stulen I., Perez-Soba M., Kok L.J.D. & van der Eerden (1998). Impacts of gaseous nitrogen deposition on plant functioning. *New Phytologist* **139**, 61-70.

Sullivan J.H. & Teramura A.H. (1992). The effects of ultraviolet-B radiation on loblolly pine. *Trees – Structure & Function* **6**, 115-120.

Sullivan J.S. (1997). Effects of increasing UV-B radiation and atmospheric CO_2 on photosynthesis and growth: implications for terrestrial ecosystems. *Plant Ecology* **128**, 194-206.

Summers C.G. & Stapleton J.J. (2002). Use of UV reflective mulch to delay the colonization and reduce the severity of *Bernisia argentifolii* (Homptera: Aleyrodidae) infestations in cucurbits. *Crop Protection* **21**, 921-928.

Summers C.G., Jeffrey M.P. & Stapleton J.J. (2004). Management of aphid borne viruses and *Bernisia argentifolii* (Homptera: Aleyrodidae) in Zucchini Squash by using reflective plastic and wheat straw mulches. *Environmental Entomology* **33**, 1447-1457.

Sumner L.W., Mendes P. & Dixon R.A. (2003). Plant metabolomics: Large-scale phytochemistry in the functional genomics era. *Phytochemistry* **62**, 817-836.

Tallowin J.R.B. & Smith R.E.N. (1994). *The effects of inorganic fertilisers in flower-rich hay meadows on the Somerset levels*. English Nature, Peterborough.

Taylor D.R. & Aarssen L.W. (1989). On the density dependence of replacement-series competition experiments. *Journal of Ecology* **77**, 975-988.

Teughels H., Nijs I., van Hecke O. & Impens I. (1995). Competition in a global change environment: The importance of different plant traits for competitive success. *Journal of Biogeography* **22**, 297-305.

Theodose T.A. & Bowman W.D. (1997). The influence of interspecific competition on the distribution of an Alpine graminoid: Evidence for the importance of plant competition in an extreme environment. *Oikos* **79**, 101-114.

Theodose T.A., Jaegar C.H., Bowman W.D. & Schardt J.C. (1996). Uptake and allocation of N-15 in alpine plants: Implications for the importance of competitive ability in predicting community structure in a stressful environment. *Oikos* **75**, 59-66.

Thiel S., Döhring T., Köfferlein M., Kosak A., Martin P. & Seidlitz H.K. (1996). A phytotron for plant stress research: how far can artificial lighting compare to natural sunlight? *Journal of Plant Physiology* **148**, 456-463.

Tilman (1990). Constraints and trade-offs – towards a predictive theory of competition and succession. *Oikos* **58**, 3-15.

Tilman D. (1985). The resource-ratio hypothesis of plant succession. *American Naturalist* **125**, 827-852.

Tilman D. (1987). The importance of mechanisms of inter-specific competition. *American Naturalist* **129**, 769-774.

Tilman D. (1999). The ecological consequences of changes in biodiversity: A search for general principles. *Ecology* **80**, 1455-1474.

Tilman D., Knops J., Wedin D., Reich P., Ritchie M. & Siemann E. (1997). The influence of functional diversity and composition on ecosystem processes. *Science* **277**, 1300-1302.

Tilman D., Reich P.B. & Knops J.M.H. (2006). Biodiversity and ecosystem stability in a decade-long grassland experiment. *Nature* **441**, 629-632.

Timmins É.M., Howell S.A., Alsberg B.K., Noble W.C. & Goodacre R. (1998). Rapid differentiation of closely related *Candida* species and strains by pyrolysis-mass spectroscopy and Fourier-transform infrared spectroscopy. *Journal of Clinical Microbiology* **36**, 367-374.

Torssell B.W. (1973). Patterns and processes in Townsville Stylo-annual grass pasture ecosystem. *Journal of Applied Ecology* **10**, 463-478.

Tosserams M., Smet J., Magendans E & Rozema J. (2001). Nutrient availability influences UV-B sensitivity of *Plantago lanceolata*. *Plant Ecology* **154**, 157-168.

Tso M.-I., Wu H.-C. & Hwang I.-R. (2005). Giant wood spider *Nephilia pilipes* alters silk protein in response to prey variation. *Journal of Experimental Biology* **208**, 1053-1061.

Tuma I., Holub P. & Fiala K. (2005). Competitive balance and nitrogen losses from three grass species (*Arrhenatherum elatius, Calamagrostis epigejos, Festuca ovina*). *Biologia* **60**, 417-422.

Turkington R. & Joliffe P.A. (1996). Interference in *Trifolium repens – Lolium perenne* mixtures: Short- and long-term relationships. *Journal of Ecology* **84**, 563-571.

Turkington R., Klein E. & Chanway C.P. (1993). Interactive effects of nutrients and disturbance – an experimental test of plant strategy theory. *Ecology* **74**, 863-878.

Twolan-Strutt L. & Keddy P.A. (1996). Above- and belowground competition intensity in two contrasting wetland plant communities. *Ecology* **77**, 259-270.

Urich-Merzenich G., Zeitler H., Jobst D., Panek D., Vetter H. & Wagner H. (2007). Application of the "-omic-" technologies in phytomedicine. *Phytomedicine* **14**, 70-82.

van de Staaij J., Rozema J., van Been A. & Aerts R. (2001). Increased solar UV-B radiation may reduce infection by arbuscular mycorrhizal fungi (AMF) in dune grassland plants: Evidence from five years of field exposure. *Plant Ecology* **154**, 169.

van de Staaij J.W.M., Bolink E., Rozema J. & Ernst W.H.O. (1997). The impact of elevated UV-B (280-320 nm) radiation levels on the reproduction biology of a highland and a lowland population of *Silene vulgaris*. *Plant Ecology* **128**, 173-179.

van der Eerden L.J. (1998). Nitrogen on microbial and global scales. *New Phytologist* **139**, 201-204.

van der Eerden L.J., Dueck T.A., Berdowski J.J.M., Greven H. & Dobben H.F. (1991). Influence of NH_3 & $(NH_4)_2SO_4$ on heathland vegetation. *Acta Botanica Neerlandica* **40**, 281-296.

van Ruijven J. & Berendse F. (2003). Positive effects of plant species diversity on productivity in the absence of legumes. *Ecology Letters* **6**, 170-175.

Varotsos C., Alexandris D., Chronopoulos G. & Tzanis C. (2001). Aircraft observations of the solar ultraviolet irradiance throughout the troposphere. *Journal of Geophysical Research – Atmospheres* **106**, 14843-14854.

Verhoef H.A., Verspagen J.M.H. & Zoomer H.R. (2000). Direct and indirect effects of ultraviolet-B radiation on soil biota, decomposition and nutrient fluxes in dune grassland soil systems. *Biology & Fertility of Soils* **31**, 366-371.

Vila M. & Weiner J. (2004). Are invasive plant species better competitors than native plant species? Evidence from pair-wise experiments. *Oikos* **105**, 229-238.

Vitousek P.M. (1994). Beyond global warming – ecology and global change. *Ecology* **75**, 1861-1876.

Want E.J., Nordstrom A., Morita H. & Siuzdak G. (2007). From exogenous to endogenous: The inevitable imprint of mass spectrometry in metabolomics. *Journal of Proteome Research* **6**, 459-468.

Wardle D.A. & Grime J.P. (2003). Biodiversity and stability of grassland ecosystem functioning. *Oikos* **100**, 622-623.

Warembourg F.R. & Estelrich H.D. (2001). Plant phenology and soil fertility effects on below-ground carbon allocation for an annual (*Bromus madritensis*) and a perennial (*Bromus erectus*) grass species. *Soil Biology & Biochemistry* **33**, 1291-1303.

Wayne P.M., Carnelli A.L., Connolly J. & Bazzaz F.A. (1999). The density dependence of plant responses to elevated CO_2. *Journal of Ecology* **87**, 183-192.

Weckworth W. (2003). Metabolomics in systems biology. *Annual Review of Plant Biology* **54**, 669-689.

Weih M., Johansson U. & Gwynn Jones D. (1998). Growth and nitrogen utilization of mountain birch (*Betula pubescens* subsp. *tortuosa*) as affected by ultraviolet radiation (UV-A and UV-B) under laboratory and outdoor conditions. *Trees Structure and Function* **12**, 201-207.

Weiher E., van der Werf A., Thompson K., Roderick M., Garnier E. & Eriksson O. (1999). Challenging Theophrastus: A common core list of plant traits for functional ecology. *Journal of Vegetation Science* **10**, 609-260.

Welker J.M., Wookey P.A., Parsons A.N., Press M.C., Callaghan T.V. & Lee J.A. (1993). Leaf carbon-isotope discrimination of vegetative responses of *Dryas octopetala* to temperature and water manipulations in a high arctic polar semi-desert, Svalbard. *Oecologia* **95**, 463-469.

Wenny B.N., Saxena V.K. & Frederick J.E. (2001). Aerosol optical depth measurements and their impact on surface levels of ultraviolet-B radiation. *Journal of Geophysical Research – Atmospheres* **106**, 17311-17319.

White A.L. & Jahnke L.S. Removing UV-A and UV-C radiation from UV-B fluorescent lamp emissions. differences in the inhibition of photosynthesis in the marine alga *Dunaliella tertiolecta* using chromate versus cellulose acetate-polyester filters. *Photochemistry & Photobiology* **80**, 340-345.

Willems J.H., Peet R.K. & Bik L. (1993). Changes in chalk grassland structure and species richness resulting from selective nutrient additions. *Journal of Vegetation Science* **4**, 203-212.

Williams J.M. ed. (2006). *Common Standards Monitoring for Designated Sites: First Six Year Report*. Peterborough, JNCC, 3556.

Willis A.J. (1963). Braunton Burrows: The effects on the vegetation of the addition of mineral nutrients to the dune soils. *Journal of Ecology* **51**, 353-374.

Wilson E.J., Wells T.C.E. & Sparks T.H. (1995). Are calcareous grasslands in the UK under threat from nitrogen deposition? An experimental determination of a critical load. *Journal of Ecology* **83**, 823-832.

Wilson J.B. & Lee W.G. (2000). C-S-R triangle theory: Community-level predictions, tests, evaluation of criticisms, and relation to other theories. *Oikos* **91**, 77-96.

Wilson S.D. & Tilman D. (1993). Plant competition and resource availability in response to disturbance and fertilisation. *Ecology* **74**, 599-611.

Winder C.L., Gordon S.V., Dale J., Hewinson R.G. & Goodacre R. (2006). Metabolic fingerprints of *Mycobacterium bovis* cluster with molecular type: Implications for genotype-phenotype links. *Microbiology* **152**, 2756-2757.

Winson M.K., Goodacre R., Woodward A.M., Timmins É.M., Jones A., Alsberg B.K., Rowland J.J. & Kell D.B. (1997). Diffuse reflectance absorbance spectroscopy taking in chemometrics (DRASTIC): A hyperspectral FT-IR-based approach to rapid screening for metabolite overproduction. *Analytica Chimica Acta* **348**, 273-282.

Woodward F.I., Smith T.M. & Emanuel W.R. (1995). A global land primary productivity and phytogeography model. *Global Biogeochemical Cycles* **9**, 471-490.

Wookey P.A., Parsons A.N., Welker J.M., Potter J.A., Callaghan T.V., Lee J.A. & Press M.C. (1993). Comparative responses of phenology and reproductive development to simulated environmental change in sub-Arctic and high-Arctic plants. *Oikos* **67**, 490-502.

Wookey P.A., Robinson C.H., Parsons A.N., Walker J.M., Press M.C., Callaghan T.V. & Lee J.A. (1995). Experimental constraints on the growth, photosynthesis and reproductive development of *Dryas octopetala* to simulated environmental change in a high arctic polar semi-desert, Svalbard. *Oecologia* **102**, 478-489.

Wright J.P., Naeem S., Hector A., Lehman C., Reich P.B., Schmid B. & Tilman D. (2006). Conventional functional classification schemes underestimate the relationship with ecosystem functioning. *Ecology Letters* **9**, 111-120.

Wu H., Prately J., Lemerie D., An M. & Liu D.L. (2007). Modern genomic approaches to improve allelopathic capability in wheat (*Triticum aestivum* L). *Allelopathy Journal* **19**, 97-107.

Xiong F.S., Ruhland C.T. & Day T.A. (2002). Effects of springtime solar ultraviolet-B radiation on growth of *Colobanthus quitensis* at Palmer Station, Antarctica. *Global Change Biology* **8**, 1146-1155.

Xu K. & Qiu B.S. (2007). Responses of superhigh-yield hybrid rice Liangyoupeijiu to enhancement of ultraviolet-B radiation. *Plant Science* **172**, 139-149.

Yan X.L., Wu P., Ling H.Q., Xu G.H., Xu F.S. & Zhang Q.F. (2006). Plant nutriomics in China: An overview. *Annals of Botany* **98**, 473-482.

Yao X.Q. & Liu Q. (2006b). Changes in morphological, photosynthetic and physiological responses of Mono Maple seedlings to enhanced UV-B and to nitrogen addition. *Plant Growth Regulation* **50**, 165-177.

Yao X.Q. & Liu Q. (2007). Changes in photosynthesis and antioxidant defenses of *Picea asperata* seedlings to enhanced ultraviolet-B and to nitrogen supply. *Physiologia Plantarum* **129**, 364-374.

Yao Y., Yang Y., Ren J. & Li C. (2006a). UV-spectra dependence of seedling injury and photosynthetic pigment change in *Cucumis sativus* and *Glycine max*. *Environmental & Experimental Botany* **57**, 160-167.

Yuan L., Ming Y., Xun Ling W. & Zhi De H. (1999). Competition and sensitivity of wheat and wild oat exposed to enhanced UV-B radiation at different densities under field conditions. *Environmental & Experimental Botany* **41**, 47-55.

Zaller J.G., Caldwell M.M., Flint S.D., Scopel A.L., Salo O.E. & Ballaré C.L. (2002). Solar UV-B radiation affects belowground parameters in a fen ecosystem in Tierra del Fuego, Argentina: Implications of stratospheric ozone depletion. *Global Change Biology* **8**, 867-871.